Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 3035–3040, March 1999
Immunology

The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-κB pathways

PATRICK S. COSTELLO, ALICE E. WALTERS, P. JOSEPH MEE, MARTIN TURNER*, LUCINDA F. REYNOLDS, ANTONELLA PRISCO, NITZA SARNER, ROSE ZAMOYSKA, AND VICTOR L. J. TYBULEWICZ†

National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, United Kingdom

Edited by Owen N. Witte, University of California, Los Angeles, CA, and approved January 13, 1999 (received for review August 24, 1998)

ABSTRACT **Vav is a GTP/GDP exchange factor (GEF) for members of the Rho-family of GTPases that is rapidly tyrosine-phosphorylated after engagement of the T cell receptor (TCR), suggesting that it may transduce signals from the receptor. T cells from mice made Vav-deficient by gene targeting (*Vav*$^{-/-}$) fail to proliferate in response to TCR stimulation because they fail to secrete IL-2. We now show that this is due at least in part to the failure to initiate *IL-2* gene transcription. Furthermore, we analyze TCR-proximal signaling pathways in *Vav*$^{-/-}$ T cells and show that despite normal activation of the Lck and ZAP-70 tyrosine kinases, the mutant cells have specific defects in TCR-induced intracellular calcium fluxes, in the activation of extracellular signal-regulated mitogen-activated protein kinases and in the activation of the NF-κB transcription factor. Finally, we show that the greatly reduced TCR-induced calcium flux of Vav-deficient T cells is an important cause of their proliferative defect, because restoration of the calcium flux with a calcium ionophore reverses the phenotype.**

Stimulation of the T cell receptor (TCR) leads to the rapid activation of tyrosine kinases that phosphorylate a variety of signal transducing proteins. These in turn activate signaling pathways, including a rise in intracellular calcium, the activation of three distinct mitogen-activated protein kinase (MAPK) cascades, and the induction of a number of transcription factors. On a longer time scale, these pathways lead to changes of gene expression, notably of cytokine genes such as *IL-2* (1).

The protooncogene Vav was discovered by virtue of a mutation that rendered it able to transform fibroblasts (2). Vav contains a domain that is similar to the protooncogene Dbl, a guanine nucleotide exchange factor (GEF) for the Rho/Rac/CDC42 family of low molecular weight Ras-like GTPases (3, 4). In addition, Vav contains a pleckstrin homology domain, a single SH2 domain, and two SH3 domains, which suggest that Vav can interact with multiple components of signal transduction pathways (5). Recent biochemical analysis as well as genetic studies in yeast have shown that Vav, when tyrosine phosphorylated, acts to promote Rac1 and other Rho-family proteins to the active GTP-bound state (6–9).

Vav is expressed at high levels in T cells and is rapidly phosphorylated by tyrosine kinases after stimulation of either the TCR or CD28, suggesting that Vav may transduce signals from either or both receptors (10–13). In support of this, Vav has been shown to regulate the transcription of genes expressed by T cells; overexpression of Vav in Jurkat T cells enhances basal and TCR-activated transcription of the *IL-2* gene and reporter constructs containing multiple NF-AT binding sites (14, 15). By using the *Rag-1*$^{-/-}$ blastocyst complementation technique, we and others found that T cell development is impaired in the absence of Vav and that mature T cells that lack Vav proliferate poorly and produce little IL-2 in response to stimulation through the TCR, suggesting that Vav plays an important role in signal transduction pathways activated by the TCR (16–18). Furthermore, we recently established a mouse strain carrying a disruption in the *Vav* gene and demonstrated that Vav was a critical signal transducer of TCR signals that drive positive and negative selection of thymocytes (19).

In this report, we have made use of Vav-deficient T cells to investigate the role of Vav in TCR-proximal signaling events. We show that, although the activation of the tyrosine kinases Lck and ZAP-70 and phosphorylation of multiple intracellular proteins is normal, the mutant T cells have specific defects in TCR-induced calcium fluxes and in the activation of the extracellular signal-regulated kinase (ERK) MAPK and NF-κB pathways.

MATERIALS AND METHODS

Mice. All strains of mice were bred at the National Institute for Medical Research. The generation of mice carrying a mutation disrupting the *Vav* gene (*Vav*$^{Tybtm1/Tybtm1}$; *Vav*$^{-/-}$) has been described (19). The IL-2 promoter-luciferase reporter transgene (IL-2Luctg) consists of the IL-2 promoter (−325 to +47 bp relative to the start of transcription) driving the expression of firefly luciferase. This construct was used to make transgenic mice in (CBA×B10)F_1 fertilized eggs by standard procedures and was maintained on a C57BL/10 background. The *Vav*$^{-/-}$/IL-2Luctg mice were on a segregating 129/Sv and C57BL/10 background; all other mice were inbred on a 129/Sv background. In all cases, mutant and control mice were age- and sex-matched and used at 8–10 weeks of age.

Proliferation Assays. Spleens were disaggregated in air-buffered Iscove's modified Dulbecco's medium (GIBCO/BRL). Single cell suspensions were incubated with anti-CD8 (YTS169), anti-class II (M5114), and anti-B220 (RA3–3A1) for 15 min on ice before the addition of "Low-Tox" Rabbit Complement (Cedarlane Laboratories) and incubation for 45 min at 37°C. The cell suspension was layered onto Lympholyte-M (Cedarlane Laboratories) and centrifuged for 20 min at 1,000 × *g*. The buffy coat was removed and washed

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: GEF, GTP/GDP exchange factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; IP_3, inositol 1,4,5-triphosphate; PLC, phospholipase C; GST, glutathione *S*-transferase; TCR, T cell receptor; RBM, RPMI/BSA medium.

*Present address: The Babraham Institute, Babraham, Cambridge CB2 4AT, United Kingdom.
†To whom reprint requests should be addressed. e-mail: v-tybule@nimr.mrc.ac.uk.

twice in PBS, the cells were labeled for 30 min with anti-CD4-PE, and $CD4^+$ T cells were sterile sorted by using a FACStar Plus flow cytometer (Becton Dickinson). Typical purity was >96%. Sorted cells were washed, resuspended in RPMI 1640 medium, 10% fetal calf serum, sodium pyruvate (1 mM), and 2-mercaptoethanol (5×10^{-5} M) (RPMI complete medium) and plated in 96-well flat-bottomed plates at 2.5×10^5 cells per ml, 0.2 ml per well. Some wells also contained immobilized anti-CD3 antibody (145.2C11), soluble anti-CD28 antibody (37–51), and ionomycin at the indicated concentrations. To measure proliferation, cells were pulsed with ^{3}H-thymidine (0.5 μCi per well; Amersham; 1 Ci = 37 GBq) and harvested after 4 hr, and incorporated radioactivity was quantitated.

Stimulation of Splenic $CD4^+$ T Cells for Biochemical Analysis. For all biochemical analysis, splenic $CD4^+$ T cells were first enriched by complement lysis and Lympholyte-M centrifugation as described above, and the $CD4^+$ T cells were purified by negative selection by using Mouse CD4 subset columns (R&D Systems, Abingdon, U.K.) according to the manufacturer's instructions. Typical purity was 90–95%. For stimulations, the cells were preincubated with anti-CD3 and anti-CD28 at 10 μg/ml for 20 min in RPMI 1640 medium and 0.1% BSA (RBM), washed, and then incubated in RBM for 5 min at 37°C before crosslinking of the antibodies with goat anti-Armenian hamster IgG antiserum (100 μg/ml; Jackson ImmunoResearch). In some cases, the cells were also stimulated with ionomycin (1 μg/ml unless otherwise indicated) or phorbol 12,13-dibutyrate (10 ng/ml).

Measurement of Luciferase Activity. $CD4^+$ splenic T cells purified as described in the previous section were stimulated for 24 hr either in RPMI complete medium alone or in the presence of immobilized anti-CD3 and soluble anti-CD28 at 1.3×10^6 cells per ml. Cells were harvested, washed in PBS, and lysed in Cell Culture Lysis Reagent (Promega). Luciferase activity was quantitated on a Clinilumat (Berthold) by using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

Immunoblotting and Immunoprecipitation. For total cytoplasmic lysates, cells were stimulated at 10^7 cells per ml in RBM, centrifuged at specified time points at $560 \times g$ for 60 s, resuspended in ice-cold lysis buffer (150 mM NaCl/20 mM Tris·Cl, pH 7.0/10 mM iodoacetamide/1% Nonidet P-40/1 mM Na_3VO_4/10 μg/ml each of chymostatin, pepstatin, and leupeptin), and cleared by centrifugation at $15{,}340 \times g$ for 20 min at 4°C. For immunoprecipitations, cells were typically challenged at 10^7 cells per ml in RBM, lysed by the addition of an equal volume of 2 × lysis buffer, and cleared by centrifugation. Immunoprecipitations, SDS/PAGE, and immunoblotting were carried out by standard procedures. For glutathione *S*-transferase (GST)–Grb2 affinity pull-downs, cells were lysed in lysis buffer containing 1% Brij in place of Nonidet P-40 and precipitations of GST–Grb2-associated proteins were carried out as described (20). PLCγ1 was immunoprecipitated by using a rabbit polyclonal antiserum (no. 06–152; Upstate Biotechnology, Lake Placid, NY) and immunoblotted with a mixture of anti-PLCγ mAbs ("Powerclone"; Upstate Biotechnology). The following antibodies were used for immunoblotting: antiphosphotyrosine antibody (4G10; Upstate Biotechnology); anti-NF-ATp rabbit antisera (1:1 mixture of α-67.1 and αT2B1; P. Hogan, Harvard Medical School) (21); anti-IκBα rabbit antiserum (C-21; Santa Cruz Biotechnology); anti-phosphoERK rabbit antiserum (anti-active MAPK; Promega); anti-Lck rabbit antiserum (no. 2166; S. Ley, National Institute for Medical Research, London); and anti-p38 rabbit antiserum (SAK7; J. Saklatvala, Kennedy Institute of Rheumatology, London). Antibody binding was revealed with goat anti-mouse IgG-horseradish peroxidase (Southern Biotechnology Associates) or protein A-horseradish peroxidase(Amersham) for monoclonals and rabbit polyclonal sera, respectively.

Intracellular Calcium Analysis. Four-color flow cytometric analysis of intracellular calcium using Indo-1 was performed as described (19).

Inositol 1,4,5-Trisphosphate (IP_3) Measurement. Purified $CD4^+$ splenic T cells were precoated with anti-CD3 and anti-CD28 antibodies and stimulated at $2.3–3 \times 10^7$ cells per ml in 130 μl of RBM by the addition of goat anti-hamster IgG crosslinking antibody (final concentration of 300 μg/ml). The stimulations were terminated by the addition of 10 μl of ice-cold 6.1 M trichloroacetic acid followed by 15-min incubation on ice. The samples were centrifuged at $1{,}400 \times g$, 4°C for 15 min, extracted with 10 volumes water-saturated diethyl ether, and neutralized with 10 μl of 1 M $NaHCO_3$, and the final volume of the aqueous phase was adjusted to 200 μl with water. IP_3 was quantitated in duplicate 100-μl samples by using a competitive [^{3}H]IP_3 binding assay (NEN) according to the manufacturer's instructions.

Electrophorectic Shift Mobility Assay. Purified splenic $CD4^+$ T cells stimulated in RBM were lysed in 20 mM Hepes (pH 7.9), 450 mM NaCl, 25% glycerol, 0.5 mM DTT, and 0.4 mM EDTA and cleared by centrifugation. Lysate containing 10 μg of protein was mixed with ^{32}P-end-labeled NF-κB oligonucleotide (Promega, E3291) and 1 μg of poly[d(I-C)] in 10 mM Hepes (pH 7.8), 60 mM KCl, 0.4 mM DTT, 10% glycerol, and 200 μg/ml BSA for 30 min at 4°C, and complexes were separated on a 7% acrylamide, 1× Tris/Borate/EDTA gel.

MAPK Assays. Stimulated T cells were lysed in lysis buffer containing 1% Triton X-100 in place of Nonidet P-40. ERK2 immunoprecipitated with a rabbit anti-ERK2 serum (C. Marshall, Institute of Cancer Research, London) was used to phosphorylate myelin basic protein using standard procedures. For p38 assays, p38 precipitated with a goat anti-p38 serum (Santa Cruz Biotechnology) was used to phosphorylate GST–ATF2 by using standard procedures (22).

RESULTS

Role of Vav in Signal Transduction from TCR/CD3 and CD28. The availability of sufficient numbers of T cells from the *Vav*$^{-/-}$ mouse strain allowed us to extend our earlier analysis to determine whether Vav transduces signals from TCR/CD3, CD28, or both. Because the Vav mutation blocks positive selection of transgenic TCRs (19), it was not possible to generate a cohort of *Vav*$^{-/-}$ T cells carrying a monoclonal TCR with a known peptide specificity. Thus, in common with many other studies, we carried out signaling experiments by using anti-CD3 antibodies to mimic the stimulation of the TCR/CD3 complex by peptide/MHC complexes. Stimulation of *Vav*$^{-/-}$ splenic $CD4^+$ T cells through CD3 alone resulted in much less proliferation than in control cells, though some proliferation was always seen at the highest doses of anti-CD3 (Fig. 1*a*). Thus Vav transduces some, though not all, of the signals from CD3 required for proliferation. Stimulation of CD28 alone cannot induce proliferation; it can only enhance proliferation in response to other stimuli (e.g., CD3). This enhancement was seen in *Vav*$^{-/-}$ $CD4^+$ T cells, suggesting that at least some CD28 signals are Vav-independent (Fig. 1*a*). However, because *Vav*$^{-/-}$ T cells have a defect in CD3 signaling, and the extent of CD28-mediated enhancement of CD3-driven proliferation is very dependent on the strength of CD3 signal (data not shown), it is impossible to determine if in the mutant cells the CD28 signals leading to proliferation are completely normal.

Vav Transduces Signals Required for IL-2 Transcription. We and others showed earlier that *Vav*$^{-/-}$ $CD4^+$ T cells secreted much less IL-2 than wild-type T cells in response to TCR stimulation (16, 17). To investigate whether this was due to a failure to initiate *IL-2* gene transcription, we crossed mice carrying the *Vav* mutation with transgenic mice containing a

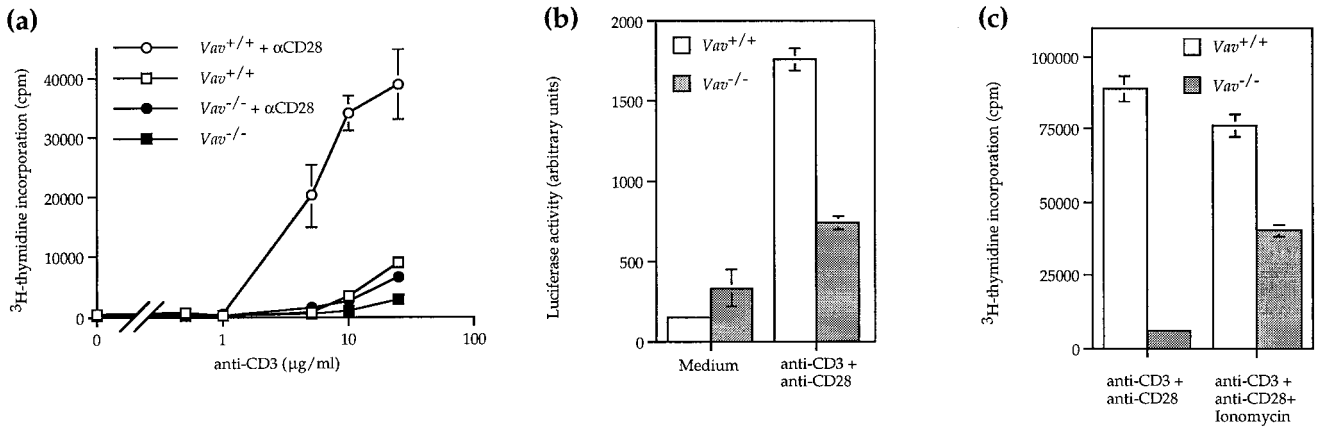

FIG. 1. TCR-induced proliferation and activation of IL-2 gene transcription in $Vav^{-/-}$ T cells. (*a*) Proliferation of $Vav^{+/+}$ or $Vav^{-/-}$ $CD4^+$ splenic T cells in response to a range of concentrations of plate-bound anti-CD3 antibody in the presence or absence of soluble anti-CD28 antibody (10 μg/ml). Proliferation was assessed by the incorporation of ^{3}H-thymidine during the final 4 hr of a 48 hr assay. Graph shows the mean ^{3}H-thymidine incorporation (±SEM) of triplicate samples. (*b*) Induction of IL-2 gene transcription assessed by the production of luciferase by $Vav^{+/+}$ or $Vav^{-/-}CD4^+$ T cells purified from mice carrying a luciferase transgene under the control of the IL-2 promoter. Cells were incubated for 24 hr either with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (10 μg/ml) or in the absence of any added stimulus (medium). (*c*) Proliferation of $Vav^{+/+}$ or $Vav^{-/-}$ $CD4^+$ T cells in response to plate-bound anti-CD3 antibody (5 μg/ml) and soluble anti-CD28 antibody (10 μg/ml) in the absence or presence of ionomycin (198 ng/ml). Proliferation was assessed as in Fig. 1*a*.

luciferase reporter gene under the control of the IL-2 promoter (IL-2Luctg). Stimulation of purified splenic $CD4^+$ T cells from $Vav^{-/-}$/IL-2Luctg mice resulted in significantly lower production of luciferase than seen with wild-type T cells (Fig. 1*b*). Thus, the *Vav* mutation results in the failure of CD3/CD28 signals to activate transcription from the IL-2 promoter.

Vav is Required for a Normal TCR-Induced Rise in Intracellular Calcium. Next we investigated the earliest TCR-proximal signaling events. TCR stimulation of mutant T cells caused normal tyrosine phosphorylation of Lck and ZAP-70, suggesting that both kinases are activated normally (data not shown). Furthermore, the tyrosine phosphorylation of phospholipase-Cγ1 (PLCγ1) and a number of Grb2-associated proteins (SLP-76, LAT, and Cbl) was normal (Fig. 2 *a* and *b*). Thus, the activation of TCR-proximal tyrosine kinases appears unaffected by the lack of Vav.

In contrast, by using flow cytometry to measure the rise in intracellular calcium, we found that $Vav^{-/-}$ $CD4^+$ splenic T cells gave either undetectable or much lower calcium fluxes than control cells (Fig. 3*a*). This result is in agreement with our previous observations on the defective TCR-induced calcium flux in $Vav^{-/-}$ thymocytes (19). However in experiments on $Vav^{-/-}$ T cells isolated from $Rag\text{-}1^{-/-}$ chimeras, we reported that the cells had normal calcium fluxes (16). This discrepancy is due to the method of cell purification: in our earlier work the T cells were isolated with anti-CD5-coated magnetic beads, which causes the cells to have near normal fluxes (data not shown). In contrast, in the experiment shown in Fig. 3*a*, the cells were not enriched in any way before analysis.

The TCR-induced rise in intracellular calcium is driven by the release of IP_3, a second messenger generated by the action of phospholipase C (PLC) on phosphatidylinositol-4,5-bisphosphate (23). $Vav^{-/-}$ T cells released much less IP_3 in response to CD3/CD28 stimulation (Fig. 3*b*), suggesting that this is likely to be the explanation for the defective calcium flux.

The NF-AT family of transcription factors, which have been implicated in the activation of the *IL-2* gene, are proteins that translocate into the nucleus under the influence of calcineurin, a calcium-activated phosphatase (1). As expected from the impaired TCR-induced calcium flux in $Vav^{-/-}$ T cells, the dephosphorylation of NF-ATp, one member of the NF-AT family, was largely blocked in the mutant cells (Fig. 4*a*), though

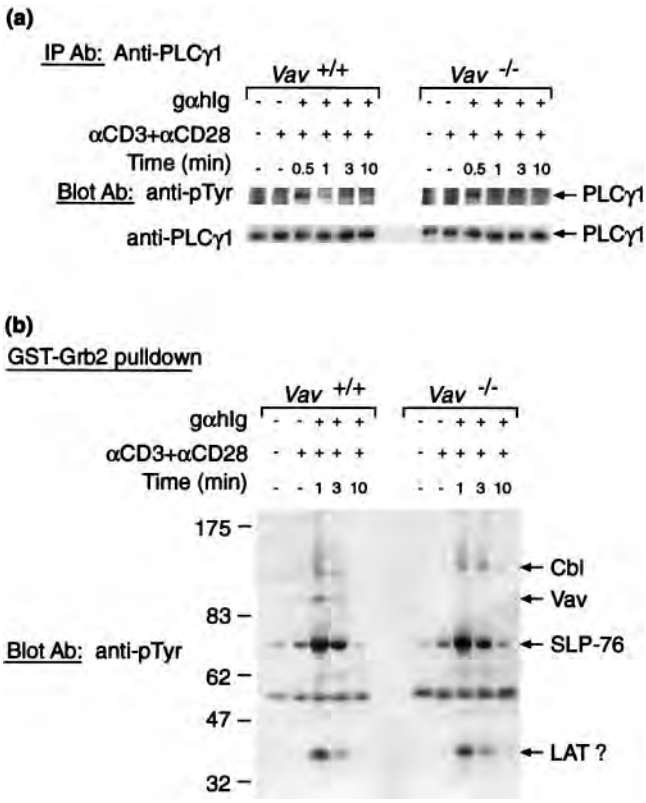

FIG. 2. TCR-induced tyrosine phosphorylation. Immunoblot of cytoplasmic extracts of $CD4^+$ splenic T cells purified from $Vav^{+/+}$ or $Vav^{-/-}$ mice. Where indicated cells were precoated with anti-CD3 and anti-CD28 antibodies (αCD3 + αCD28) that in some samples were then crosslinked with goat anti-hamster Ig polyclonal antiserum (gαhIg; 100 μg/ml) and samples taken after the indicated time. (*a*) Samples were immunoprecipitated (IP) with antibodies to PLCγ1, and analyzed by immunoblotting with an antibody to phosphotyrosine (pTyr) and then stripped and reprobed with an antibody to PLCγ1. (*b*) Proteins binding to a GST–Grb2 fusion protein were isolated from stimulated cell extracts and analyzed by immunoblotting with an anti-pTyr antibody. The identity of individual pTyr-containing proteins was suggested by reprobing the blot with antibodies to Cbl, Vav, and SLP-76 (data not shown). The 36-kDa phosphoprotein is likely to be LAT (38). Sizes are shown in kDa.

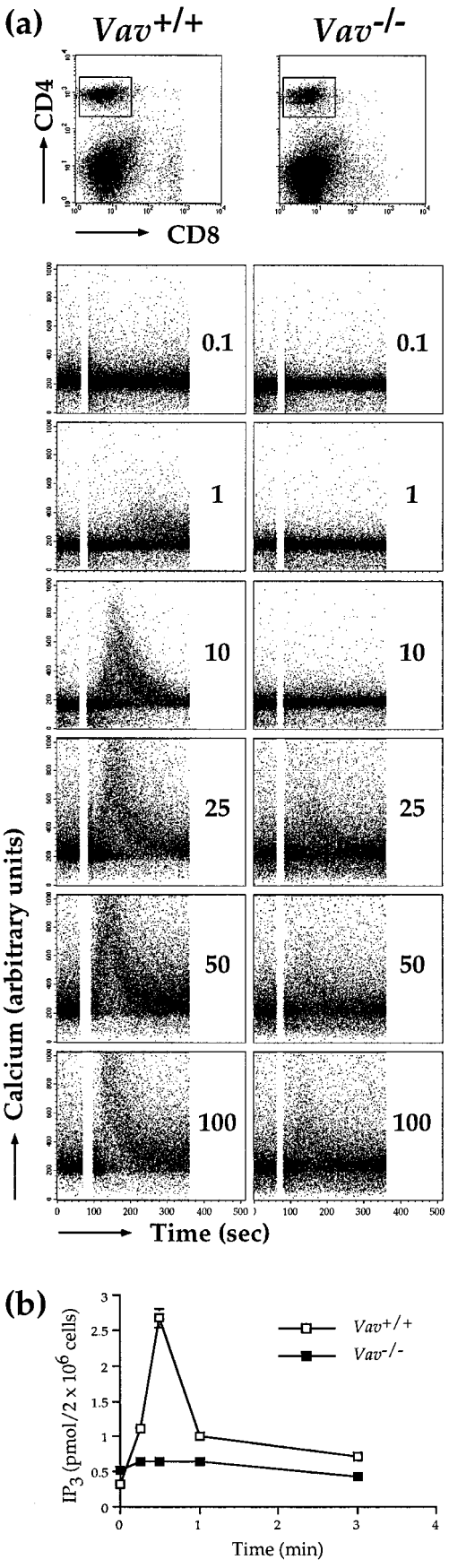

FIG. 3. Release of intracellular calcium and IP_3. (*a*) Intracellular calcium flux is reduced in Vav-deficient $CD4^+$ splenic T cells. The two panels at the top show flow cytometric analysis of CD4 and CD8 staining of Vav-deficient ($Vav^{-/-}$) or control ($Vav^{+/+}$) splenocytes preloaded with Indo-1 and coated with anti-CD3. Panels below show intracellular calcium concentrations in the $CD4^+$ T cells (gated as shown in the CD4/CD8 plots) as a ratio of Indo-1 violet/blue fluorescence versus time. Cells were stimulated with 0.1–100 μg/ml goat anti-hamster antibody to crosslink the anti-CD3 at the time indicated by the break in the calcium trace. Adequate loading of the $Vav^{-/-}$ T cells with Indo-1 was demonstrated by inducing a calcium flux with ionomycin (not shown). (*b*) Graph showing mean levels of IP_3 (±SEM) in $CD4^+$ T cells precoated with anti-CD3 and anti-CD28 antibodies and stimulated by the addition of gαIg (300 μg/ml).

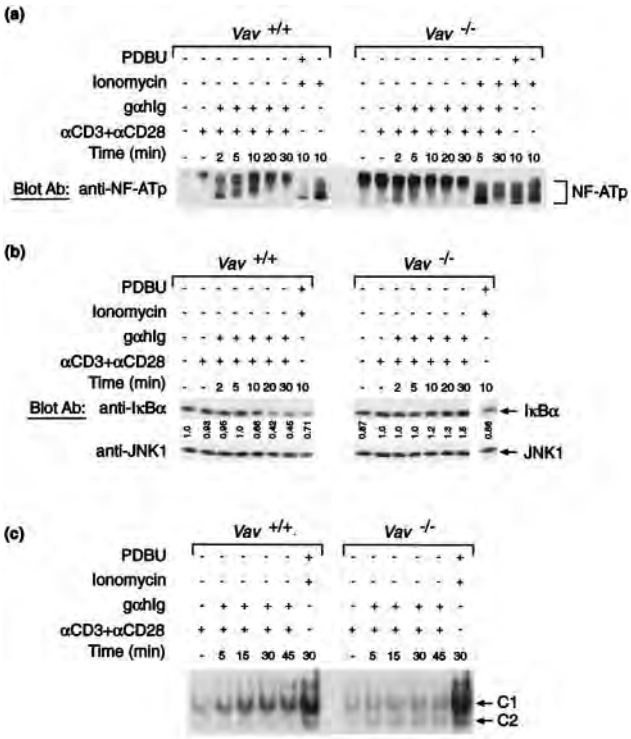

FIG. 4. NF-AT and NF-κB pathways in $Vav^{-/-}$ T cells. Immunoblot of cytoplasmic extracts of $CD4^+$ splenic T cells stimulated as described in Fig. 2. In addition in some cases the cells were also stimulated with ionomycin (500 ng/ml) or phorbol 12,13-dibutyrate (PDBU; 10 ng/ml). (*a*) Immunoblot probed with an antiserum to NF-ATp. Dephosphorylation of NF-ATp results in a faster relative electrophoretic mobility and lower apparent molecular weight. (*b*) Immunoblot probed with an anti-IκBα antiserum and reprobed with an anti-JNK1 antibody to control for loading. Numbers below the IκBα blot represent relative amounts of IκBα normalized to JNK1 in each lane and to the amount of IκBα in the first lane (unstimulated $Vav^{+/+}$ cells). Degradation of IκBα in TCR-stimulated $Vav^{+/+}$ $CD4^+$ T cells can be seen in the disappearance of the IκBα band. (*c*) NF-κB complexes visualized by electrophoretic mobility shift assay using ^{32}P-labeled NF-κB probe bound to extracts from $Vav^{+/+}$ or $Vav^{-/-}$ $CD4^+$ splenic T cells. Based on data in the literature (24) and on our own experiments using anti-p50 and anti-p65 antibodies to supershift these complexes (data not shown), C1 contains both p50 and p65, whereas C2 contains p50, but not p65.

some dephosphorylation was always visible (Fig. 4*a*; $Vav^{-/-}$ T cells, 2 min), presumably as a result of the residual calcium flux. Treatment of $Vav^{-/-}$ T cells with ionomycin, a calcium ionophore that directly induces an intracellular calcium flux, rescues the defect in NF-ATp dephosphorylation, consistent with the suggestion that it was due to the reduced calcium flux (Fig. 4*a*).

In view of this result we asked whether ionomycin could rescue the proliferative defect of $Vav^{-/-}$ T cells. Stimulation of $Vav^{-/-}$ and control $CD4^+$ T cells through CD3 and CD28 in the presence of ionomycin rescued much though not all of the proliferative defect of the mutant cells (Fig. 1*c*) and suggested

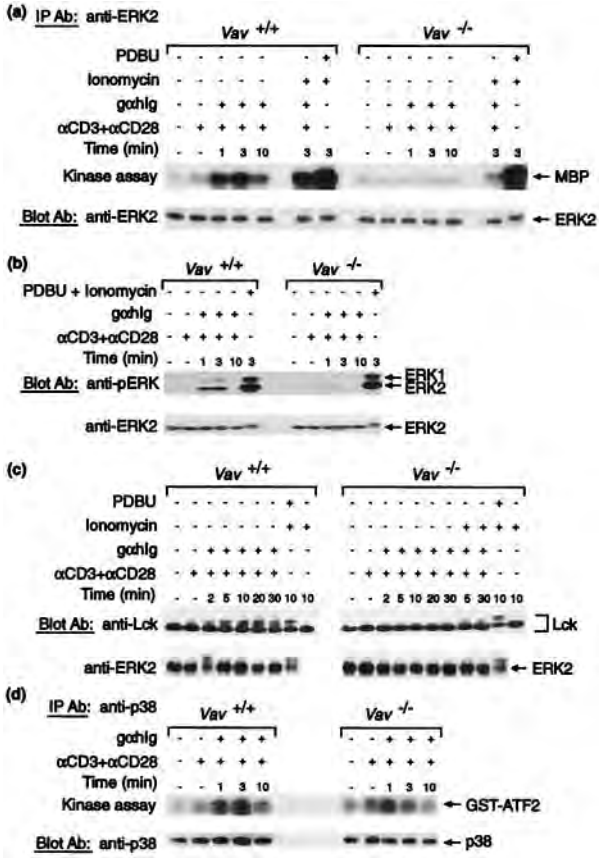

FIG. 5. Activation of MAPK pathways. $CD4^+$ splenic T cells purified from $Vav^{+/+}$ or $Vav^{-/-}$ mice were stimulated as described in Figs. 2 and 4. (*a*) ERK2 MAPK was immunoprecipitated from stimulated cells and used in an *in vitro* kinase assay with ^{32}P-ATP to phosphorylate myelin basic protein (MBP). (*Upper*) An autoradiograph of a blot showing phosphorylation of myelin basic protein. In the lower panel the same blot has been probed with an anti-ERK2 antibody to control for loading. (*b*) Immunoblot of stimulated cell extracts probed with an anti-phosphoERK antibody and reprobed with an anti-ERK2 antibody to control for loading. Phosphorylation of the ERK1 and ERK2 kinases is indicative of their activation by the MEK kinase. (*c, Upper*) an immunoblot of stimulated cell extracts probed with an anti-Lck antibody. The shift in mobility of Lck to a slower migrating form of higher apparent molecular weight is caused by serine phosphorylation, which is likely to be downstream of ERK activation (26). Tyrosine phosphorylation of Lck does not cause this mobility shift. This shift is readily seen on 7–15% acrylamide gradient gels. (*c, Lower*) A blot of the same samples probed with an anti-ERK2 antibody showing the phosphorylation-induced mobility shift. In this experiment ionomycin was used at 500 ng/ml. These shifts are only seen upon extended electrophoresis; they were not seen in *a* and *b* as electrophoresis times were too short. (*d*) p38 MAPK was immunoprecipitated from stimulated cells and used in an *in vitro* kinase assay with ^{32}P-ATP to phosphorylate a GST–ATF2 fusion protein. (*Upper*) An autoradiograph of the blot showing phosphorylation of GST–ATF2. In the lower panel the same blot has been probed with an anti-p38 antibody to control for loading.

that the abnormal calcium flux is an important cause of the reduced cellular proliferation. Nonetheless, because the rescue was not complete, there are likely to be other Vav-dependent TCR signaling pathways in addition to the calcium flux.

Vav Transduces Signals to the NF-κB Pathway. T cells from mice deficient in c-Rel, a member of the NF-κB family of transcription factors, fail to produce IL-2 and to proliferate in response to TCR stimulation, demonstrating that these factors are also important regulators of *IL-2* transcription (24). We monitored the activation of NF-κB by the degradation of the inhibitor subunit IκB, a necessary prerequisite for the activation of NF-κB. In contrast to wild-type T cells, CD3/CD28 stimulation of $Vav^{-/-}$ splenic T cells resulted in no degradation of IκBα (Fig. 4*b*). Furthermore, direct analysis of NF-κB transcription factors by electrophoretic mobility shift assay demonstrated that TCR engagement resulted in little or no induction of NF-κB activity in $Vav^{-/-}$ cells (Fig. 4*c*). TCR-induced activation of NF-κB in T cells requires increased levels of intracellular calcium (25); however, addition of ionomycin could not rescue the defect in IκBα degradation (data not shown), suggesting again that there must be other Vav-dependent TCR-induced pathways in addition to calcium flux.

TCR-Induced ERK Kinase Activation is Defective in $Vav^{-/-}$ T Cells. TCR stimulation has been reported to cause activation of the ERK, JNK, and p38 MAPKs (1). An *in vitro* kinase assay showed that, in contrast to $Vav^{+/+}$ cells, CD3/CD28 stimulation of $Vav^{-/-}$ $CD4^+$ T cells resulted in little or no visible induction of ERK2 activity (Fig. 5*a*). Immunoblotting with an antibody specific for the phosphorylated, active forms of the ERKs showed that whereas phosphorylated ERK1 and ERK2 were readily detectable in $Vav^{+/+}$ T cells, only a very small quantity of phosphorylated ERK2 was seen in the mutant T cells (Fig. 5*b*). This phosphorylation can also be monitored as a mobility shift on SDS/PAGE and was visible in wild-type but not mutant T cells (Fig. 5*c*). TCR stimulation results in the serine phosphorylation of Lck, which is likely to be dependent on the activity of ERKs (26) and can be seen as a mobility shift to more slowly migrating isoforms. Wild-type but not mutant T cells showed a clear Lck mobility shift (Fig. 5*c*). Taken together these data clearly demonstrate that the TCR-induced activation and phosphorylation of the ERK kinases is defective in $Vav^{-/-}$ T cells. Addition of ionomycin to CD3/CD28-stimulated $Vav^{-/-}$ T cells resulted in a small increase in ERK activity (Fig. 5 *a* and *c*). However, because this rescue is only partial, another Vav-dependent pathway must also be involved in the activation of ERKs in addition to calcium flux.

In contrast to the results on ERK activation, we found that in both wild-type and $Vav^{-/-}$ $CD4^+$ T cells, p38 MAPK activity was induced to a similar extent (Fig. 5*d*).

DISCUSSION

Our results demonstrate that Vav transduces TCR/CD28 signals to the calcium, ERK, and NF-κB pathways. The calcium defect is most likely due to the greatly reduced production of IP_3 (Fig. 3*b*) and may reflect reduced activity of PLCγ1, an enzyme regulated by Lck, ZAP-70, and Itk tyrosine kinases and by phosphatidylinositol-3-OH kinase (27). The activation of the Lck and ZAP-70 kinases appears unaffected in the mutant T cells, as is the tyrosine phosphorylation of PLCγ1. However, tyrosine phosphorylation is not sufficient to activate PLCγ1; it also needs the 3-phosphorylated lipid products of phosphatidylinositol-3-OH kinase for full activation (28). Because Vav binds to the p85 subunit of phosphatidylinositol-3-OH kinase (5) and thus may regulate its activity, this could be a mechanism by which Vav controls the function of PLCγ1, IP_3 production, and hence the calcium flux.

Alternatively, Vav may modulate the availability of phosphatidylinositol-4,5,-bisphosphate, the substrate for PLCγ1. The Rho-family of GTPases has been implicated in the regulation of phosphatidylinositol-4-phosphate-5-kinase leading to increased production of phosphatidylinositol-4,5,-bisphosphate (29, 30). A failure to activate phosphatidylinositol-4-phosphate-5-kinase in Vav-deficient T cells would lead to a shortage of phosphatidylinositol-4,5,-bisphosphate, decreased production of IP_3 and hence a diminished calcium flux. Such a hypothesis is given support by studies of $Vav^{-/-}$ B cells in which CD19-induced phosphatidylinositol-4-phosphate-5-kinase activation is greatly reduced (31). Furthermore, we note a recent report has shown that the Rac-1 GTPase regulates NF-AT dephosphorylation, consistent with the possibility of a

Vav/Rac-1 pathway, which regulates calcium flux and hence the dephosphorylation of NF-AT via the calcium-activated phosphatase calcineurin (32).

How might Vav regulate ERK MAPKs? It has been claimed that Vav is a GEF for Ras, which could explain the Vav-dependence of TCR-induced ERK activation (5). However, most reports now agree that Vav is more likely to be a GEF for Rho-family GTPases (8, 9). These have been proposed to activate a cascade consisting of PAK1, MEK1, and ERK kinases (33). If such a pathway exists in mouse T cells, it could explain the failure of TCR-induced ERK activation in $Vav^{-/-}$ T cells.

The degradation of IκB is triggered by its phosphorylation by IκB kinases (34). This pathway may be regulated by Rho-family GTPases via the activation of MEKK1 kinase (34). If such a pathway is used by the TCR, Vav, by virtue of its GEF activity for Rho-family GTPases may activate MEKK1 and IκB kinase and thus signal the degradation of IκB.

Our results do not exclude the possibility that Vav may transduce other signals. $Vav^{-/-}$ T cells fail to form an actin-dependent TCR cap that may transduce signals required for the induction of *IL-2* transcription (35, 36). Cytochalasin D blocks actin polymerization and inhibits the formation of these caps, but does not interfere with early TCR-proximal signaling events such as calcium flux and ERK and JNK activation (36, 37). Thus, the defects in TCR-induced calcium and ERK activation in $Vav^{-/-}$ T cells cannot be a consequence of defective cap formation; rather they must lie on pathways upstream of cap induction or on parallel unrelated pathways.

There are a couple of apparent discrepancies between our paper and those of Fischer *et al.* and Holsinger *et al.* (35, 36). Fischer *et al*. (35) report no defect in CD3/CD28-induced ERK activation in $Vav^{-/-}$ T cells. We measured ERK activation in several different ways (see Fig. 5), and each experiment was carried out at least three times, giving the same result on all occasions. The discrepancy may be due to the different mutation made by Fisher *et al*. that may not have removed all of Vav's function. Alternatively, the difference may be a result of strain differences; all of our experiments were carried out by using mice inbred on 129/Sv background, whereas Fisher *et al*. used the outbred CD1 mouse strain. Holsinger *et al.* (36) report that $Vav^{-/-}$ T cells can translocate NF-ATc1 into the nucleus, in apparent contradiction with our observation that very little NF-ATp is dephosphorylated. We note that in our experiments we always saw a small amount of NF-ATp dephosphorylation (Fig. 4*a*) in $Vav^{-/-}$ T cells, though always a lot less than in control cells. Perhaps this small amount of dephosphorylated NF-ATp is sufficient to give the NF-AT translocation observed by Holsinger *et al*. Finally, we note that in this paper the authors use yet another different *Vav* mutation, which may perhaps have retained some residual Vav function.

In conclusion, we have shown that Vav, a GEF for Rho-family GTPases, transduces TCR signals to calcium, ERK, and NF-κB pathways and thus lies in a pivotal position in TCR signal transduction. The addition of ionomycin to TCR-stimulated $Vav^{-/-}$ T cells rescues much of their proliferative defect. Hence, the abnormal TCR-induced calcium flux in Vav-deficient T cells is an important cause of their greatly diminished IL-2 production and proliferation.

We thank C. Atkins for flow cytometric analysis; S. Ley, H. Coope, M. Belich, R. Huby, T. Ahmad, M. Walmsley, E. Schweighoffer, and D. Cantrell for advice; S. Ley, A. Magee, C. Marshall, J. Tite, A. Weiss, P. Hogan, and J. Saklatvala for gifts of antibodies; and J. Downward, S. Taga, and D. Cantrell for GST fusion proteins. We thank G. Crabtree for communication of results before publication. This work was supported by the Medical Research Council and a grant from the Leukaemia Research Fund (V.T.).

1. Cantrell, D. (1996) *Annu. Rev. Immunol.* **14,** 259–274.
2. Katzav, S., Martin-Zanca, D. & Barbacid, M. (1989) *EMBO J.* **8,** 2283–2290.
3. Adams, J. M., Houston, H., Allen, J., Lints, T. & Harvey, R. (1992) *Oncogene* **7,** 611–618.
4. Boguski, M. S., Bairoch, A., Attwood, T. K. & Michaels, G. S. (1992) *Nature (London)* **358,** 113.
5. Collins, T., Deckert, M. & Altman, A. (1997) *Immunol. Today* **18,** 221–225.
6. Crespo, P., Bustelo, X. R., Aaronson, D. S., Coso, O. A., Lopez-Barahona, M., Barbacid, M. & Gutkind, J. S. (1996) *Oncogene* **13,** 455–460.
7. Olson, M. F., Pasteris, N. G., Gorski, J. L. & Hall, A. (1996) *Curr. Biol.* **6,** 1628–1633.
8. Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S. & Bustelo, X. R. (1997) *Nature (London)* **385,** 169–172.
9. Han, J., Das, B., Wei, W., Van Aelst, L., Mosteller, R. D., Khosravi Far, R., Westwick, J. K., Der, C. J. & Broek, D. (1997) *Mol. Cell. Biol.* **17,** 1346–1353.
10. Bustelo, X. R., Ledbetter, J. A. & Barbacid, M. (1992) *Nature (London)* **356,** 68–71.
11. Margolis, B., Hu, P., Katzav, S., Li, W., Oliver, J. M., Ullrich, A., Weiss, A. & Schlessinger, J. (1992) *Nature (London)* **356,** 71–74.
12. Nunes, J. A., Collette, Y., Truneh, A., Olive, D. & Cantrell, D. A. (1994) *J. Exp. Med.* **180,** 1067–1076.
13. August, A., Gibson, S., Kawakami, Y., Kawakami, T., Mills, G. B. & Dupont, B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9347–9351.
14. Holsinger, L. J., Spencer, D. M., Austin, D. J., Schreiber, S. L. & Crabtree, G. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9810–9814.
15. Wu, J., Katzav, S. & Weiss, A. (1995) *Mol. Cell. Biol.* **15,** 4337–4346.
16. Tarakhovsky, A., Turner, M., Schaal, S., Mee, P. J., Duddy, L. P., Rajewsky, K. & Tybulewicz, V. L. J. (1995) *Nature (London)* **374,** 467–470.
17. Zhang, R., Alt, F. W., Davidson, L., Orkin, S. H. & Swat, W. (1995) *Nature (London)* **374,** 470–473.
18. Fischer, K. D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A. & Guidos, C. (1995) *Nature (London)* **374,** 474–477.
19. Turner, M., Mee, P. J., Walters, A., Quinn, M. E., Mellor, A. L., Zamoyska, R. & Tybulewicz, V. L. J. (1997) *Immunity* **7,** 451–460.
20. Buday, L., Egan, S. E., Rodriguez Viciana, P., Cantrell, D. A. & Downward, J. (1994) *J. Biol. Chem.* **269,** 9019–23.
21. Ho, A. M., Jain, J., Rao, A. & Hogan, P. G. (1994) *J. Biol. Chem.* **269,** 28181–28186.
22. Gupta, S., Campbell, D., Derijard, B. & Davis, R. J. (1995) *Science* **267,** 389–393.
23. Berridge, M. J. (1993) *Nature (London)* **361,** 315–325.
24. Köntgen, F., Grumont, R. J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D. & Gerondakis, S. (1995) *Genes Dev.* **9,** 1965–1977.
25. Kanno, T. & Siebenlist, U. (1996) *J. Immunol.* **157,** 5277–5283.
26. Watts, J. D., Sanghera, J. S., Pelech, S. L. & Aebersold, R. (1993) *J. Biol. Chem.* **268,** 23275–23282.
27. Scharenberg, A. M., El-Hillal, O., Fruman, D. A., Beitz, L. O., Li, Z., Lin, S., Gout, I., Cantley, L. C., Rawlings, D. J. & Kinet, J.-P. (1998) *EMBO J.* **17,** 1961–1972.
28. Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A. & Schlessinger, J. (1998) *EMBO J.* **17,** 414–422.
29. Chong, L. D., Traynor Kaplan, A., Bokoch, G. M. & Schwartz, M. A. (1994) *Cell* **79,** 507–513.
30. Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A. & Stossel, T. P. (1995) *Cell* **82,** 643–653.
31. O'Rourke, L., Tooze, R., Turner, M., Sandoval, D. M., Carter, R. H., Tybulewicz, V. L. J. & Fearon, D. T. (1998) *Immunity* **8,** 635–645.
32. Turner, H., Gomez, M., McKenzie, E., Kirchem, A., Lennard, A. & Cantrell, D. A. (1998) *J. Exp. Med.* **188,** 527–537.
33. Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E. & Cobb, M. H. (1997) *EMBO J.* **16,** 6426–6438.
34. May, M. J. & Ghosh, S. (1998) *Immunol Today* **19,** 80–88.
35. Fischer, K.-D., Kong, Y.-Y., Nishina, H., Tedford, K., Marengère, L. E. M., Kozieradzki, I., Sasaki, T., Starr, M., Chan, G., *et al.* (1998) *Curr. Biol.* **8,** 554–562.
36. Holsinger, L. J., Graef, I., Swat, W., Chi, T., Bautista, D. M., Davidson, L., Lewis, R. S., Alt, F. W. & Crabtree, G. R. (1998) *Curr. Biol.* **8,** 563–572.
37. Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. (1995) *J. Exp. Med.* **181,** 577–584.
38. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P. & Samelson, L. E. (1998) *Cell* **92,** 83–92.