Vav1 Transduces T Cell Receptor Signals to the Activation of the Ras/ERK Pathway via LAT, Sos, and RasGRP1*

Received for publication, January 9, 2004, and in revised form, January 28, 2004
Published, JBC Papers in Press, February 5, 2004, DOI 10.1074/jbc.M400257200

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Vav1 is a signaling protein required for both positive and negative selection of mature CD4⁺CD8⁺ double positive thymocytes. Activation of the ERK MAPK pathway is also required for positive selection. Previous work has shown that Vav1 transduces T cell receptor (TCR) signals leading to an intracellular calcium flux. We now show that in double positive thymocytes, Vav1 is required for TCR-induced activation of the ERK1 and ERK2 kinases via a pathway involving the Ras GTPase, and B-Raf, MEK1, and MEK2 kinases. Furthermore, we show that Vav1 transduces TCR signals to Ras by controlling the membrane recruitment of two guanine nucleotide exchange factors. First, Vav1 transduces signals via phospholipase Cγ1 leading to the membrane recruitment of RasGRP1. Second, Vav1 is required for recruitment of Sos1 and -2 to the transmembrane adapter protein LAT. Finally, we show that Vav1 is required for TCR-induced LAT phosphorylation, a key event for the activation of both phospholipase Cγ1 and Sos1/2. We propose that reduced LAT phosphorylation is the key reason for defective TCR-induced calcium flux and ERK activation in Vav1-deficient cells.

Signals from the T cell receptor (TCR) play a critical role in the positive and negative selection of immature CD4⁺CD8⁺ double positive (DP) thymocytes, which lead to the development of mature T cells. The precise outcome of these selection events is determined by signals generated following interactions between the TCR on DP thymocytes and peptides presented by MHC class I and class II molecules on thymic stromal cells (1). Cells whose TCR binds with no or low avidity to peptide-MHC complexes undergo apoptosis in a process termed death by neglect. Cells bearing a TCR with moderate avidity for peptide-MHC complexes undergo apoptosis in a process termed death by neglect. The activation of the ERK MAPK pathway is thus required for both positive and negative selection in DP thymocytes (12, 13). Analysis of signaling pathways showed that in DP thymocytes, Vav1 transduces TCR signals to the induction of an intracellular calcium flux and to the activation of NF-κB and the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signaling cascade (14). More recently we have shown that in DP thymocytes, Vav1 transduces TCR signals to an intracellular calcium flux by regulating the activation of phospholipase Cγ1 (PLCγ1) via phosphoinositide 3-kinase (PI3K)-dependent and -independent pathways (15). In particular, we showed that Vav1 controls the activation of Tec family kinases such as Itk that may directly phosphorylate and activate PLCγ1. In contrast, the mechanism by which Vav1 transduces TCR signals to the ERK MAPK pathway remains unclear.

The activation of ERK MAPK in many cell types, including T cells, is believed to be under the control of the Ras GTPase. Activation of Ras leads in turn to the activation of a cascade consisting of the Raf, MEK, and ERK kinases. A number of studies have postulated that the ERK MAPK cascade is important in positive selection of DP thymocytes. Expression of dominant negative forms of H-Ras, Raf-1, or MEK1 or gene targeting of ERK1 leads to defective positive but not negative selection (16–20). However, this has been disputed by a more recent study (21) reporting defective negative selection in thymocytes treated with a MEK inhibitor. Nonetheless, an understanding of how TCR signaling in DP thymocytes results in ERK activation would be an important contribution to elucidating how TCR signals lead to positive and negative selection.

In this study, we examine how Vav1 transduces TCR signals in DP thymocytes to the activation of the ERK MAPK cascade, and we show that Vav1 is required for the activation of the Ras...
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GTase and its downstream ERK MAPK pathway. Vav1 is shown to be required for TCR regulation of two distinct GEFs for Ras. We demonstrate first that Vav1 transduces phosphorylation of LAT on tyrosine residues that play important roles in the activation of both PLCy1 and Sos1 and -2.

EXPERIMENTAL PROCEDURES

Mice—The generation of mice carrying a mutation disrupting the Vav1 gene (Vav1(12iTyv/Y12iTyv); Vav1(−/−)) as well as F5 Rag1−/−β-Jm−/− and Vav1−/−F5Rag1−/−β-Jm−/− mice have been described previously (12, 15, 22).

Stimulation of Thymocytes for Biochemical Analysis—For all biochemical analyses, thymi were disaggregated in air-buffered IMDM. For TCR stimulation, cells were preincubated with the hamster anti-mouse CD3ε monoclonal antibody (2C11; 10 μg/ml; Pharmingen) on ice for 30 min, washed, and then incubated in air-buffered IMDM for 5 min at 37 °C prior to cross-linking of the antibodies with goat anti-Armenian hamster IgG antiserum (75 μg/ml; Jackson Immunol. Research). For stimulations with phorbol 12,13-dibutyrate (10 ng/ml) or ionomycin (1 μg/ml), cells were not pretreated with anti-CD3ε. For studies with inhibitors, thymocytes were preincubated with the inhibitors at 37 °C for 30 min prior to standing on ice for 10 min. Preincubation with anti-CD3ε on ice then proceeded as above, still in the presence of inhibitor. Subsequent cross-linking of anti-CD3ε also occurred in the presence of inhibitor. For control samples where no inhibitor was used, an equal volume of PBS-0.1% BSA carrier was added. The following inhibitors were used: U73122 (1 μM), H9262 and H9280 (10 μM), BAPTA-AM (15 μM) and BAPTA-AM (15 μM) (both from Calbiochem).

Immunoblotting of Total Cell Lysates—All chemicals were obtained from Sigma unless otherwise indicated. Cells were typically stimulated at 1 × 10^6 cells/ml in air-buffered IMDM for the specified times, and stimulation was stopped by the addition of an equal volume of 2X RIPA lysis buffer (2% Igepal CA-630, 100 mM Tris-Cl, pH 7.5, 300 mM NaCl, 0.2% SDS, 10 mM EDTA, 20 mM NaF, 20 mM dithiothreitol, 2 mM sodium orthovanadate, 1.5 mM sodium deoxycholate, 50 mM Tris-Cl, pH 7.5, 0.5 mM MgCl2, 50 mM NaCl, 1 nM BAPTA-AM, and 10 μg/ml BAPTA-AM (both from Calbiochem). Cells were cleared by centrifugation at 15,340 × g for 10 min at 4 °C. An equal volume of 2× Laemmli carrier was added, and the samples were heated at 95 °C for 3 min. SDS-PAGE and immunoblotting were carried out by standard procedures. The following antibodies were used for immunoblotting: anti-phospho-ERK (E4), anti-RasGRP1, anti-B-Raf, anti-Pak, anti-Grb2, anti-Sos1, and anti-Sos2 (all from Santa Cruz Biotechnology); anti-phospho-MEK1/2, anti-MEK1/2, anti-phosphoerine 144-Pak1, anti-phospho-epidermal growth factor receptor (EGFR)-185-C, and anti-phosphothreonine 538-PKCa, and anti-phosphotyrosine 175-murine LAT (equivalent to pY175 murine LAT; all from Cell Signaling Technology); anti-Ras (Ras10), anti-phosphotyrosine 191-human LAT (equivalent to pY195 murine LAT), and anti-phosphotyrosine 226-human LAT (equivalent to pY226 human LAT; all from Upstate Biotechnology, Inc.); anti-PDK1-horseradish peroxidase (Transduction Laboratories), anti-ERK2 (gift from C. Marshall, London), anti-LAT (gift from M. Turner, Bahram Institute), anti-phospho-PKD, and anti-PKD (gifts from D. Cantrell, Dundee, Scotland, UK). Antibody binding was revealed with goat anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) for mouse monoclonal antibody and goat anti-rabbit IgG-horseradish peroxidase (Cell Signaling Technology) or protein A-horseradish peroxidase (Amersham Biosciences) for rabbit polyclonal antibody.

B-Raf Kinase Assay—Stimulated cell suspensions were lysed in an equal volume of 2× B-Raf Buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 2% Triton X-100, 0.2% SDS, 1 mM EDTA, 0.05% 2-mercaptoethanol, 2 mM sodium orthovanadate, 1.5 mM sodium deoxycholate, and 20 mM NaF). The lysates were incubated for 90 min at 4 °C, centrifuged, and the supernatant was adjusted to a final concentration of 150 mM NaCl, 5 mM EDTA. Insoluble material was pelleted by centrifugation at 15,340 × g at 4 °C for 10 min. The supernatant was then incubated with 1 μg of monoclonal antibody (anti-B-Raf) for 1 h at 4 °C, followed by the addition of protein A-sepharose 4B, washed three times with cold phosphate-buffered saline, 5 mM MgCl2, 0.1% Triton X-100, and bound protein was eluted with 2× Laemmli sample buffer that had been preheated to 95 °C and analyzed by immunoblotting for Ras.

Cell Fractionation—Stimulated thymocytes were centrifuged at 15,340 × g for 10 s at 4 °C, resuspended in Hypotonic Buffer (10 mM Tris-Cl, pH 7.5, 0.5 mM MgCl2, 50 mM NaF, 1 nM sodium orthovanadate, 1.100 (v/v) mammalian cell protease inhibitor mixture), and incubated 10 min at 4 °C. Cells were lysed using 20 strokes of an Eppendorf micropestle, and debris was removed by centrifugation at 2000 × g for 5 min, and the supernatant was adjusted to a final concentration of 150 mM NaCl, 5 mM EDTA. Insoluble material was pelleted by centrifugation at 15,340 × g for 15 min at 4 °C, and an equal volume of 2× Laemmli sample buffer was added, and the samples were heated at 95 °C for 3 min.

Immunoprecipitation of Grb2—Stimulated thymocytes were lysed in 2× BOG Buffer (2% β-octyl glucopyranoside, 2% Triton X-100, 100 mM Tris-Cl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 20 mM NaF, 20 mM sodium orthovanadate, 20 mM sodium dithiothreitol, 20 mM sodium orthovanadate, and 10 μg/ml BAPTA-AM (both from Calbiochem). Cells were lysed and centrifuged at 15,340 × g for 10 min at 4 °C. Anti-Grb2 antibody was added, and the lysate was incubated for 1 h at 4 °C, followed by protein A-Sepharose and incubation overnight at 4 °C. Immunoprecipitates were washed five times in 1× BOG buffer, and bound protein was eluted using 2× Laemmli Buffer at 95 °C for 5 min by immunoblotting.

RESULTS

Vav1 Is Required for TCR-induced Activation of Ras, B-Raf, MEK, and ERK—to investigate the mechanism by which Vav1 might transduce signals to the Ras/ERK pathway in DP thymocytes, we made use of our previously described genetic system which allows access to a uniform population of DP thymocytes (15, 22). The system consists of the F5 TCR transgene that expresses a class I-restricted TCR specific for a peptide...
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From influenza nuclear protein presented by H-2Db on a background deficient in Rag-1 (Rag-1−/−) and MHC class I (β2m−/−). In the resultant FSReg−1−/−β2m−/− mice, the absence of class I molecules causes a complete block in positive selection and thus all the F5 TCR-expressing thymocytes are blocked at the DP stage, just prior to positive (or negative) selection. Thymocytes from these mice were compared directly to thymocytes from Vav1−/− and Vav1−/− DP thymocytes, which was almost absent in Vav1−/− cells (Fig. 1C). Finally, we examined the activation of Ras by measuring levels of Ras-GTP (25). Whereas TCR stimulation resulted in an increase in Ras activation in Vav1−/− DP thymocytes, this was greatly reduced in Vav1−/− cells (Fig. 1D). Taken together our results show that Vav1 is required for normal TCR-induced activation of Ras, B-Raf, MEK, and ERK in DP thymocytes.

**TCR-induced Activation of Pak1 Is Not Required for ERK Activation**—Although it seems likely that the defective activation of ERK in Vav1−/− cells is because of a failure to activate Ras, we considered whether Vav1 may transduce signals to the ERK pathway via B-Raf or MEK, independently of Ras activation. One such possibility could be through the Pak kinases, because Pak1 had been shown to phosphorylate MEK1 and hence enhance its association with either Raf-1 (26) or ERK2 (27), whereas Pak2 has been reported to phosphorylate and thus enhance the activation of Raf-1 (28). Because Vav1 is a GEF for Rac1 and Rac2 GTPases, which in turn activate Pak kinases, we hypothesized that Vav1 may transduce signals to the ERK pathway via Rac and Pak. We had shown previously (15) that TCR-induced Rac1 activation is impaired in Vav1−/− DP thymocytes. Consistent with this, TCR-induced phosphorylation of Pak1 on its autophosphorylation sites (serines 144, 199, and 204) (29) was greatly reduced in Vav1−/− cells (Fig. 2A), suggesting that its activation is also defective. Similar studies showed no detectable TCR-induced phosphorylation of Pak2 in wild-type or mutant cells (not shown).

To evaluate directly the contribution of Pak kinases to TCR-induced ERK activation, we made use of a fusion protein Tat-PID, consisting of the Pak inhibitory domain (PID) of Pak1 fused to a peptide from the human immunodeficiency virus Tat protein that can direct the transduction of proteins across plasma membranes (30). Incubation of Vav1−/− DP thymocytes with Tat-PID, resulted in an inhibition of TCR-induced Pak1 phosphorylation and, by inference, its activation (Fig. 2B). In contrast, treatment of cells with Tat-PID had no effect on TCR-induced ERK activation (Fig. 2B). We conclude that in DP thymocytes Pak1 does not transduce TCR signals to the ERK pathway.

Because Pak kinases have also been implicated in the regulation of the c-Jun NH2-terminal kinase and p38 MAP kinase pathways (31–33), we examined whether these might be defective in Vav1-deficient DP thymocytes. Although we were unable to detect TCR-induced activation of c-Jun NH2-terminal kinase in Vav1−/− DP thymocytes (not shown), we detected a small increase in p38 activation, as measured by phosphorylation...
Phosphorylation of Pak1 and ERK1/2 was observed for phosphothreonine 180 and phosphotyrosine 182 of p38 (\(p\)-p38). Treatment of Pak1 was analyzed by immunoblotting with an antiserum specific for either phosphoserines 199 and 204 of Pak1 (\(pS199/204\)-Pak1) or phosphoserine 182 of p38 (\(p\)-p38). Phosphorylation of Pak1 and ERK1/2 was evaluated as in A and Fig. 1A, respectively. C, phosphorylation of p38 MAP kinase was analyzed by immunoblotting total cell lysates with an antiserum specific for phosphothreonine 180 and phosphotyrosine 182 of p38.

Vav1 Transduces TCR Signals to Ras and ERK via PLC and DAG—TCR-induced activation of Ras within T cells is thought to be catalyzed by the GEFs RasGRP1 and Sos1 and -2. RasGRP1 is a Ras GEF whose translocation to the plasma membrane, and hence activation, is regulated by DAG, a second messenger produced by the PLC-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (34, 35). Thymocytes from RasGRP1-deficient mice are defective in TCR-induced ERK phosphorylation, suggesting that RasGRP1 is an important Ras activator downstream of the TCR and PLC (36). Sos1 and Sos2 are Ras GEFs that are recruited to the plasma membrane of T cells through the adapter protein Grb2, which binds to phosphotyrosine residues on LAT, a transmembrane adapter protein that is rapidly phosphorylated following TCR stimulation (37–40).

In view of the defective TCR-induced activation of PLC\(\gamma\)1 in Vav1-deficient thymocytes (15), it was reasonable to hypothesize that the defective Ras and ERK activation might be caused by a failure to activate RasGRP1. To evaluate this, we initially asked whether in DP thymocytes PLC is required to transduce TCR signals to the Ras/ERK pathway. Consistent with previous reports in the human Jurkat T cell line (41), both the phosphorylation of ERK and the activation of Ras were reduced by treatment with U73122 to a level similar to that observed in Vav1\(^{–/–}\) cells (Fig. 3, A and B).

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate and DAG, and inositol 1,4,5-trisphosphate in turn causes the intracellular calcium flux. We performed a number of experiments to distinguish which of these second messengers transduces TCR signals to the Ras/ERK pathway in DP thymocytes. In contrast to the effects of U73122, treatment of wild-type DP thymocytes with BAPTA, which blocks the intracellular calcium flux by chelating Ca\(^{2+}\), had no effect on TCR-induced ERK phosphorylation (Fig. 3C). Furthermore, ionomycin, which induces a calcium flux, was unable to restore ERK phosphorylation in Vav1\(^{–/–}\) cells. In contrast, treatment of Vav1\(^{–/–}\) cells with phorbol 12,13-dibutyrate, a DAG analog, resulted in phosphorylation of ERK, presumably by activating RasGRP1 and hence Ras, B-Raf, and MEK (Fig. 3D).

Defective TCR-induced DAG Production in Vav1-deficient Cells—To verify that Vav1 was required for TCR-induced DAG production, we examined the activation of protein kinase C\(\theta\) (PKC\(\theta\)), an enzyme that is activated by DAG (42). We found that while in wild-type cells TCR stimulation resulted in a large increase in phosphorylation of threonine 538 on PKC\(\theta\), this was greatly reduced in the absence of Vav1 (Fig. 4A). Thr-538 is located in the activation loop of PKC\(\theta\), and its phosphorylation is required for activation (43). PKD is an enzyme that is also responsive to DAG (44). In addition it may be directly activated by PKC\(\theta\), because inhibition of PKC blocks TCR-induced PKD phosphorylation in human T cells, and expression of a constitutively active PKC\(\theta\) results in increased PKD activity (45, 46). Consistent with a defect in DAG production and PKC\(\theta\) activation, we found that TCR stimulation leads to robust phosphorylation of PKD on serine 916 in wild-type.
thymocytes, whereas this was greatly reduced in Vav1−/− cells (Fig. 4B). Ser-916 is an autophosphorylation site on PKD that correlates with its activation (47). Furthermore, TCR-induced pS916-PKD was greatly diminished by treatment with the PLC inhibitor U73122 or with the PKC inhibitor Ro 31-8220 (Fig. 4C and data not shown). Taken together these experiments indicate that Vav1 is required to transduce signals leading to the activation of PKC and PKD, consistent with a defect in DAG production in Vav1-deficient cells.

Vav1 Is Required for TCR-induced Translocation of RasGRP1—Next we examined the role of Vav1 in TCR-induced translocation of RasGRP1 to an insoluble fraction of the cell (P100) that includes the plasma membrane, because this is the only known biochemical change in RasGRP1 that correlates with its activation (41). While in Vav1−/− DP thymocytes, TCR stimulation resulted in the translocation of RasGRP1 to the P100 fraction, and in Vav1-deficient cells, TCR stimulation typically caused little or no increase in RasGRP1 in this fraction (Fig. 5A). In addition, we consistently noted an elevated level of RasGRP1 in the P100 fraction of unstimulated Vav1−/− cells. The reasons for this are unknown, but they do not result in increased levels of Ras or ERK activation (Fig. 1, A and D).

To verify that the translocation of RasGRP1 to the P100 fraction was dependent on PLC, we made use of the PLC inhibitor U73122. As expected, treatment of wild-type DP thymocytes with U73122 inhibited the TCR-induced translocation of RasGRP1 to the P100 fraction (Fig. 5B). Most interesting, inhibitor-treated Vav1−/− cells resembled Vav1-deficient cells in having elevated levels of RasGRP1 in the P100 fraction in unstimulated cells. Thus our results suggest that Vav1 transduces TCR signals to Ras activation via PLC, DAG, and RasGRP1.

Vav1 Transduces Signals Leading to LAT Phosphorylation and Hence Membrane Recruitment of Sos1 and Sos2—Finally, we examined whether Vav1 may transduce TCR signal...
Defective translocation of RasGRP1 to the membrane in Vav1−/− thymocytes. Vav1−/− and Vav1+/− DP thymocytes were stimulated as described in Fig. 1, and then fractionated. A, the P100 fraction, containing membrane and cytoskeletal compartments, was analyzed by immunoblotting (IB) for RasGRP1. Equal loading was established by immunoblotting for LAT, which is found constitutively in the P100 fraction. B, RasGRP1 translocation to the P100 fraction was measured in Vav1−/− thymocytes stimulated in the presence or absence of the PLC inhibitor U73122.

DISCUSSION

We have shown that Vav1 is required for TCR-induced activation of ERK in DP thymocytes. In view of the proposed role of the ERK cascade in positive selection of thymocytes (16–20), this may explain in part the defective positive selection seen in Vav1−/− mice (12, 13). In contrast, negative selection of thymocytes has been linked to the activation of the c-Jun NH2-terminal kinase and p38 MAPK pathways (49–51). Given that negative selection is also defective in Vav1−/− mice (12, 13), it was surprising to find that TCR-induced p38 activation was increased in Vav1-deficient DP thymocytes. This shows that hyperactivation of p38 is not sufficient to cause negative selection and that another Vav1-dependent pathway must be involved in negative selection. This may be the ERK pathway itself, in view of recent results showing that ERK can also play a role in negative selection (21).

Our results suggest that Vav1 transduces TCR signals to ERK most probably by activating the signaling cascade Ras → B-Raf → MEK1 and -2 → ERK1 and -2 (Fig. 7). We were unable to detect any TCR-induced activation of Raf-1 in Vav1−/− cells, suggesting different functions for these Raf isoforms. Most interesting, recent results have shown that in a chicken B cell line, B cell receptor-induced ERK activation is more dependent on B-Raf rather than Raf-1 (52). We considered the possibility that Vav1 may control signaling inputs into the ERK cascade other than through Ras. Because Pak1 had been shown to phosphorylate MEK1 and hence enhance its association with either Raf-1 (26) or ERK2 (27), and is itself activated by Ral1, it was possible that Vav1 transduced TCR signals via Ral1 and Pak1 to MEK1 and ERK. However, although TCR-induced Ral1 activation (15) and Pak1 phosphorylation was defective in Vav1−/− DP thymocytes, we showed that inhibition of Pak1 did not affect ERK activation. This difference in wiring of signaling pathways may be due to different cell types or stimuli used, because the reported connections between Pak1 and MEK1 were identified in the human embryonic kidney 293 cell line or in COS-1 cells stimulated with epidermal growth factor or fibronectin, respectively (26, 27).

Another possible pathway by which Vav1 may transduce signals to ERK independently of Ras is via PKC enzymes. Studies in human T cells have pointed to the possibility that PKC enzymes might transduce TCR signals to ERK, independently of Ras (53, 54). Recent work has shown that the PKCζ is required in primary murine B cells for B cell receptor-induced ERK activation (55). By analogy, PKCζ or another PKC may have the same function in T lineage cells. Preliminary experiments using PKC inhibitors show a partial inhibition of TCR-induced ERK phosphorylation in DP thymocytes, although we
cannot exclude that these inhibitors may be having effects on other unknown targets. Our data show that Vav1 may contribute to such a PKC-mediated pathway, because the phosphorylation of PKCα is defective in Vav1-deficient DP thymocytes, in agreement with previous work showing that Vav1 is required for TCR-induced membrane translocation of PKCα in T cells (56).

The ability of Vav1 to transduce TCR signals to the activation of Ras is not direct, but rather appears to involve both RasGRP1 and the Sos family of Ras GEFs. Our studies show that in DP thymocytes Vav1 is required for TCR-induced translocation of RasGRP1 to a P100 fraction that includes the plasma membrane. Because we can show that in these cells TCR-induced activation of Ras and ERK was dependent on PLC and DAG, it seems very likely that the defective RasGRP1 translocation and hence Ras and ERK activation in Vav1−/− cells is secondary to a failure to activate PLCγ1 (Fig. 7). In agreement with this model, a recent report (57) shows that in a chicken B cell line Vav3 transduces B cell receptor signals to ERK activation via PLCγ2, DAG, and RasGRP1. The same report also suggests that Vav3 controls RasGRP1 and hence ERK activation via the induction of actin polymerization. This pathway does not seem to operate in primary murine DP thymocytes, because treatment of cells with cytochalasin D to block actin polymerization did not inhibit TCR-induced ERK phosphorylation (data not shown).

The most surprising finding of these studies is that in addition to transducing signals to RasGRP1, Vav1 also controls the membrane recruitment and thus presumably activation of the Sos1 and Sos2 Ras GEFs (Fig. 7). The failure of Sos1 and -2 to bind to LAT is most likely due to defective phosphorylation of Tyr-175, Tyr-195, and Tyr-235. When phosphorylated, these tyrosine residues bind the SH2 domain of the Grb2 adapter protein that is constitutively associated with Sos1 and -2. Although it is clear from gene targeting experiments that RasGRP1 is important in transducing TCR signals to the activation of ERK, presumably via Ras (36), the precise contributions of Sos1 and -2 remain unclear and await studies with T cells deficient in Sos1, Sos2, or both (58–60).

In addition to defective phosphorylation of LAT on Tyr-175, Tyr-195, and Tyr-235, preliminary studies show that TCR-induced phosphorylation of Tyr-136-LAT may also be reduced in Vav1−/− DP thymocytes.2 These results stand in marked contrast to our previously published study (15) where we showed that total TCR-induced tyrosine phosphorylation of LAT was unaffected by a deficiency in Vav1. Repeated experiments have confirmed this finding. The explanation for this must be that the antibody we used to detect total phosphoryrosine (RC20) only sees a subset of phosphotyrosines on LAT, and in particular does not seem to be sensitive to the level of phosphorylation on Tyr-136, Tyr-175, Tyr-195, and Tyr-235, but rather detects phosphorylation of other tyrosines. We note that in human Jurkat T cells expressing a mutant form of LAT (4YF), where Tyr-132, Tyr-171, Tyr-191, and Tyr-226 (the human equivalents of the mouse residues) were mutated to phenylalanine, TCR stimulation still resulted in detectable tyrosine phosphorylation of LAT when assessed using an antibody recognizing total phosphotyrosine (4G10) (48).

The reduced TCR-induced phosphorylation of these four tyrosine residues on LAT in Vav1−/− DP thymocytes may explain not only the decreased recruitment of Sos1 and -2 but also the reduced phosphorylation and activation of PLCγ1 and the defective calcium flux we reported earlier (15). Human Jurkat T cells expressing the 4YF LAT mutant have been found to be completely defective in TCR-induced calcium flux and to have greatly reduced ERK activation, a phenotype similar to that of Vav1-deficient primary murine DP thymocytes (48). These studies suggested that when phosphorylated, Tyr-136 of LAT binds an SH2 domain of PLCγ1, whereas phosphorylated Tyr-175, Tyr-195, and Tyr-235 bind the SH2 domains of Grb2 and the Grb2-related adapter protein Gads. As discussed earlier, Grb2 is constitutively associated with Sos1 and -2, whereas Gads binds to the adapter protein SLP-76. Normal recruitment of PLCγ1 to LAT has been shown to require both Tyr-136 as well as the distal three tyrosines (Tyr-175, Tyr-195, and Tyr-235) (48). This may be because PLCγ1 is recruited to LAT by at least two interactions. First it binds via an SH2 domain to pY136-LAT, and second it binds via an SH3 domain to a polyproline motif in SLP-76 (61), which itself is recruited via Gads to one or more of the distal three tyrosines. Thus defective phosphorylation of these four tyrosine residues on LAT in Vav1−/− DP thymocytes would be expected to result in a failure to recruit PLCγ1, SLP-76, and Gads to a LAT-nucleated complex, resulting in reduced phosphorylation and activation of PLCγ1 and a defective calcium flux. Consistent with this, we

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**Fig. 7. Diagram of Vav1-regulated pathways leading to the activation of the Ras/ERK pathway in DP thymocytes.** Vav1 transduces TCR signals to the activation of PLCγ1 via two independent routes (15). First, signals from Vav1 activate PI3K, possibly via Rac1. PI3K in turn is required for the activation of Tec family kinases such as Itk, which phosphorylate PLCγ1. Second, Vav1 is required for the phosphorylation of LAT and hence the assembly of a LAT-nucleated complex containing PLCγ1 and the adapter proteins SLP-76 and Gads. Vav1 transduces signals to the activation of Ras by controlling the plasma membrane recruitment of two distinct GEFs. First, the activation of PLCγ1 results in the production of the second messenger DAG which recruits RasGRP1 to the plasma membrane. Second, by regulating the phosphorylation of LAT on Tyr-175, Tyr-195, and Tyr-235, Vav1 is required for the formation of a LAT-Grb2-Sos1/2 complex and hence the recruitment of the Sos GEFs to the plasma membrane. Activated Ras leads to the activation of a kinase cascade involving B-Raf, MEK1 and -2, and ERK1 and -2. DAG may also activate this pathway at the level of B-Raf via PKC and PKD enzymes. Finally, we have shown that neither a calcium flux nor the Rac1-activated Pak1 kinase are required for TCR-induced ERK activation. Inhibitors referred to in the text are shown in gray.
showed previously that the TCR-induced association of SLP-76 and PLCγ1 with each other is greatly reduced in the absence of Vav1, whereas the association of both of these proteins with LAT is partially affected (15).

It remains unclear how Vav1 controls the phosphorylation of LAT. One possibility is that this may be through Tec family kinases such as Itk, whose phosphorylation is defective in Vav1−/− T cells (15). Alternatively, because Vav1 is recruited via its SH2 domain to a phosphotyrosine on SLP-76 (62–64), it may affect the assembly of a LAT/Gads/SLP-76/PLCγ1 complex by allosteric means. Decreased assembly of such a complex in Vav1−/− cells may allow increased access for protein-tyrosine phosphatases to the phosphotyrosines on LAT, thus resulting in decreased levels of LAT phosphorylation.

In conclusion, our data show that Vav1 transduces TCR signals to the activation of the Ras/Raf/MEK/ERK cascade by at least two pathways (Fig. 7). First, Vav1 is required to activate PLCγ1, generate DAG, and recruit RasGRP1 to the membrane. Second, Vav1 is required for the recruitment of Sos1 and -2 to the LAT adapter protein. Furthermore, our studies show that Vav1 is required for the TCR-induced phosphorylation of four key tyrosines on LAT, and we suggest that this may be the critical defect in Vav1−/− cells leading both to compromised calcium flux and defective ERK activation.

Acknowledgments—We thank D. Kioussis, L. Vanes, and M. Lovatt for help and advice; M. Turner, C. Marshall, and D. Cantrell for antibodies; K. Williams, R. Zamoyska, and L. Allen for help with mouse husbandry; S. Lewis, R. Zamoyska, and C. Trigueros for critical reading of the manuscript.

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J. Biol. Chem. 2004, 279:18239-18246.
doi: 10.1074/jbc.M400257200 originally published online February 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400257200

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