

Vav Is Required for Cyclin D2 Induction and Proliferation of Mouse B Lymphocytes Activated via the Antigen Receptor*

Received for publication, June 8, 2001, and in revised form, August 24, 2001
Published, JBC Papers in Press, September 6, 2001, DOI 10.1074/jbc.M105305200

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B lymphocytes from mice null for the Rho-family guanine-nucleotide exchange factor, Vav, are defective in their ability to proliferate in response to BCR cross-linking, but are able to proliferate normally in response to LPS. In addition, they have a depletion of CD5⁺ (B1) lymphocytes and defective IgG class switching. This phenotype is reminiscent of that observed in mice null for the cell cycle regulatory protein, cyclin D2. We demonstrate here that the inability of *vav*^{-/-} B cells to proliferate in response to BCR ligation is due to an inability to induce cyclin D2. In addition, we show that the proliferative defect of these cells occurs after the cells have entered early G₁ phase. Analyses of potential downstream signaling intermediates revealed differential activation of the stress-activated MAP kinases in the absence of Vav, normal activation of the ERK, MAPK, and phosphatidylinositol 3-kinase pathways, and defective intracellular calcium mobilization. We further demonstrate that intracellular calcium homeostasis is required for cyclin D2 induction, implicating a possible link with the defective calcium response of *vav*^{-/-} B cells and their inability to induce cyclin D2.

The *vav* proto-oncogene encodes a hemopoietic-specific protein that functions as a Rho-family guanine-nucleotide exchange factor (GEF)¹ (for reviews, see Refs. 1–3). Vav is rapidly phosphorylated following stimulation of the antigen receptors in B and T lymphocytes and integrates signals from antigen receptors and co-stimulatory molecules (CD19 in B cells and CD28 in T cells) (4, 5). Several protein-interaction domains are present in Vav, including the GEF catalytic Dbl domain, a Src homology 2 (SH2) domain, two SH3 domains, a calponin homology domain, and a pleckstrin homology domain.

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¹ The abbreviations used are: GEF, guanine-nucleotide exchange factor; CDK, cyclin-dependent kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; [Ca²⁺]_i, intracellular [Ca²⁺].

Analyses of *vav*^{-/-} mice have demonstrated an important role for Vav in lymphocyte development and proliferation (6, 7). Defects in T cell selection in the thymus, and in the production of peripheral T cells were observed in Vav-deficient mice (8). Vav is essential for T cell receptor capping and actin polymerization (9, 10), while in addition, T cells from Vav-deficient mice fail to proliferate in response to antigen-receptor cross-linking. A failure of interleukin-2 production by Vav-deficient T cells was demonstrated to be the molecular basis for this proliferative defect.

Recent data shows that Vav has an important role in setting the threshold for antigen receptor-mediated activation of B lymphocytes, which is likely due to its regulation of receptor clustering (11). Thus, increased degrees of antigen receptor cross-linking could partially reverse the proliferative defect in the anti-IgM response of *vav*^{-/-}-deficient B cells. Vav is also implicated in efficient T help-dependent IgG class switching, and in antibody responses to T cell-dependent haptens. In addition, Vav-deficient mice exhibit a defect in the development of peritoneal CD5⁺ (B1) B lymphocytes, a phenotype also observed in mice lacking the Vav-interacting CD19 (12, 13) or Bruton's tyrosine kinase (14) proteins. However, Vav is not essential for CD40, interleukin-4, or LPS-induced B cell activation, and *vav*^{-/-} mice mount normal B cell responses to T cell-independent repetitive viral and polyvalent hapten antigens.

Cell proliferation requires successful transition through cell cycle check-points. The D-type cyclins (cyclins D1, D2, and D3) are rate-limiting for transition through the restriction point (R) (15–17). Once cells have passed through R, the E-type cyclins (E1 and E2) accumulate and allow S phase initiation (18). The D- and E-families of G₁ cyclins have different specificities for their cyclin-dependent kinase (CDK) subunits; the D-type cyclins preferentially bind to and activate CDKs-4 and -6, while cyclin E is preferentially associated with CDK2. D-type cyclins are predominantly regulated by extracellular mitogenic signals, whereas cyclin E is regulated autonomously, in a cyclical manner, peaking at the G₁/S boundary. The primary physiological substrate for the CDKs are the retinoblastoma (pRb) family of pocket proteins (pRb, p107, and p130) (19, 20). When hypophosphorylated, the pocket proteins bind to and inactivate the E2F family of transcription factors which are required for the transcription of genes that are necessary for entry into S-phase of the cell cycle. The activities of the CDKs are negatively regulated by two families of CDK inhibitors, namely the WAF/CIP family (comprising p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) and the INK4 family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d}).

The WAF/CIP family inhibits a broad range of cyclin-CDK complexes, while the INK4 family specifically inhibit cyclin D-CDK4/6 complexes (21).

We have shown previously, that cyclin D2 is critical for IgM mediated activation of B cells and that cyclin D3 can partially compensate for the function of cyclin D2 in *cyclin D2*-deficient B cells (22). Moreover, while *cyclin D2*-deficient B cells respond poorly to weak cross-linking of the BCR by monoclonal anti-IgM, they respond normally to LPS (23). The molecular basis for the differential activation by IgM and LPS is due to specific targeting of cyclin D2 by IgM, while LPS normally activates both cyclins D2 and D3. Mice null for cyclin D2 share phenotypic features with *vav*^{-/-} mice with respect to defects in activation of B cells in response to BCR cross-linking, impaired IgG class-switching, and having a significant depletion of CD5 (B1) lymphocytes (23). In the present study, we identify Vav as a key component of IgM induced cyclin D2 induction and subsequent cell cycle progression. In addition, we begin to delineate the signaling pathways converging on cyclin D2 upon BCR cross-linking.

EXPERIMENTAL PROCEDURES

Isolation of B Cells and Cell Culture—The generation of mice homozygous for the Vav null mutation have been described previously (8). Splenic B cells were purified from these Vav null mice and their wild-type counterparts by previously published methods (24). In brief, T cells were killed by a mixture of monoclonal antibodies against Thy-1, CD4, and CD8, plus guinea pig complement. Small, dense B cells were then isolated on Percoll density gradients. These preparations typically consisted of 90–95% B-lymphocytes. The cells were cultured at 10⁶ cells ml⁻¹ in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 units ml⁻¹ penicillin/streptomycin and 50 μM 2-mercaptoethanol, and were stimulated with 10 μg ml⁻¹ of monoclonal anti-IgM (clone b.7.6) (25), anti-CD40 (clone 3/23) (26), polyclonal anti-IgM (affinity-purified F(ab')₂ goat anti-mouse purchased from Cappel/ICN), or lipopolysaccharide (LPS) (*Escherichia coli*, serotype O55:B5) (Sigma). The p38 MAPK inhibitor SB 203580 (purchased from Calbiochem) was used at 10 μM, and the calcium flux inhibitor SK&F 96365 (purchased from Sigma) at 10 μg ml⁻¹.

Cell Proliferation Analysis—Cell proliferation was monitored by [³H]thymidine incorporation assays. The cells were cultured in quadruplicate at 10⁵ cells/well in 200-μl cultures in supplemented RPMI 1640 medium, and [³H]thymidine was added for the final 4 h of a 72-, 48-, or 24-h culture period. Cells were collected using a PHD cell harvester (Cambridge Technology, Cambridge, MA) and [³H]thymidine incorporation into DNA was quantified by scintillation counting. All presented data are mean ± S.D. (*n* = 4) (some data points the error bars are smaller than the data point symbol).

Cell Cycle Analysis—Cell cycle analyses were performed by combined PI and fluorescein isothiocyanate staining as described (27). Briefly, cells were washed with phosphate-buffered saline and fixed in 90% ethanol, 10% phosphate-buffered saline. Following fixation, cells were washed again and then incubated with 500 μg ml⁻¹ DNase-free RNase, 20 μg ml⁻¹ PI, and 0.05 μg ml⁻¹ fluorescein isothiocyanate for 30 min at 37 °C prior to analysis using a FACScan flow cytometer (Becton Dickinson).

Western Blot Analysis and Antibodies—Western blot whole cell extracts were prepared by lysing cells with 4 times packed cell volume of lysis buffer (1% Nonidet P-40, 100 mM NaCl, 20 mM Tris, pH 7.4, 10 mM NaF, 1 mM sodium orthovanadate and protease inhibitors ("Complete" purchased from Roche Molecular Biochemicals) on ice for 15 min. Protein yield was quantified by Bio-Rad Dc protein assay kit (Bio-Rad). 50 μg of lysate was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and recognized by specific antibodies. The antibodies against cyclin D2 (M-20), cyclin D3 (18B6-10), cyclin E (M-20), CDK2 (M2), CDK4 (C-22), CDK6 (C-21), and p27 (C-19) were purchased from Santa Cruz Biotechnology. The anti-phospho pRB (Ser^{807/811}) antibody was purchased from New England Biolabs and the anti-phospho pRB (Thr⁸²¹) antibody from BIOSOURCE. Antibodies raised against phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), phospho-p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴), phospho SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵), phospho-AKT (Ser⁴⁷³), and antibodies raised against the total (activation state-independent) forms of these kinases were purchased from New England Biolabs. The antibodies were detected using horseradish

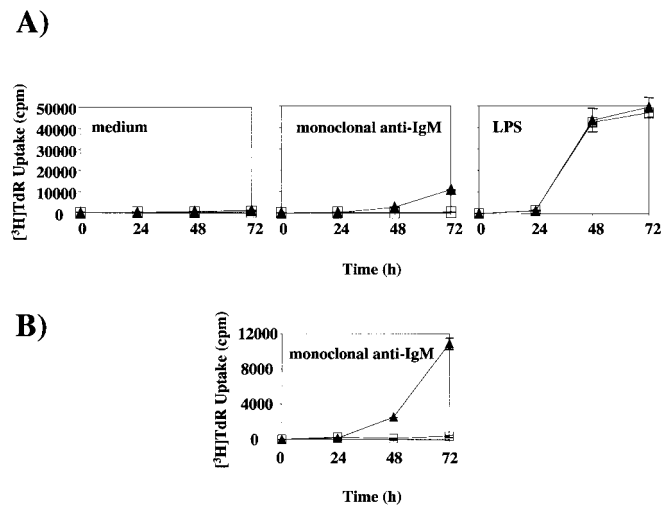


FIG. 1. Proliferation of B cells from *vav*^{-/-} and wild-type mice. A, [³H]thymidine incorporation was used to monitor S-phase entry in cultures of small dense B cells from *vav*^{-/-} (open squares) and wild-type mice (closed triangles) following treatment with either monoclonal anti-IgM (10 μg/ml b.7.6), LPS (10 μg/ml), or with no stimulation. Each data point represents a mean of four independent experiments (± S.D.). B shows a larger scale graph of cells treated with monoclonal anti-IgM (data as described for panel A).

peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako), or mouse absorbed goat anti-rat IgG (Southern Biotechnology Associates, Inc.) and visualized by the enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech, United Kingdom).

Intracellular Calcium Analysis—Small dense B cells were stained with anti-B220-fluorescein isothiocyanate before being incubated with Indo-1 acetoxyethyl ester (Indo-1) for 20 min at 37 °C. Cells were then washed before analysis of real-time intracellular free calcium by flow cytometry. Indo-1 emission was analyzed for 1 min before stimulation to establish baseline calcium concentrations, and following stimulation with monoclonal or polyclonal anti-IgMs, for a further 9 min.

RESULTS

Cell Cycle Analysis of B cells from *vav*^{-/-} and Wild-type Mice—Previous studies have shown that antigen receptor-mediated proliferative responses of B cells from *vav*^{-/-} mice are severely compromised. As a first step in identifying potential cell-cycle regulatory events responsible for this defect, we analyzed the kinetics of S-phase entry in cultures of small, dense B cells stimulated with either monoclonal anti-IgM (a relatively weak mitogen) or with the polyclonal activator, LPS (a potent mitogen). Our results showed that small, dense B cells from *vav*^{-/-} mice proliferated equally as well as those from wild-type mice in response to LPS, but unlike their wild-type counterparts, were completely unable to proliferate in response to monoclonal anti-IgM (Fig. 1). We next sought to analyze the kinetics of cell cycle entry of these cells. This was monitored by flow cytometric analysis of both DNA and protein content (Fig. 2 and Table I). Resting B cells were found to have a 2N DNA content and low protein content in both wild-type and Vav-null B cells, indicating that most cells (96 and 94%, respectively) were in G₀. Both wild-type and Vav null B cells had begun to enter G₁ by 24 h after LPS treatment, as evidenced by an increase in their protein content. At 48 and 72 h following LPS treatment, both wild-type and *vav*^{-/-} B cells were observed to have entered S and G₂/M phases, since they had a high protein content and an increased DNA content. As expected, the kinetics of cell cycle entry were slower in wild type cells stimulated with anti-IgM (compared with those activated by LPS), with cells present in G₁ phase by 48 h and S phase by 72 h. Interestingly, Vav-null cells began to enter G₁ phase by 48 h and 72 h, but did not progress into S phase. Rather, they appear to have undergone apoptosis, indicated by a sub-2N DNA content.

FIG. 2. Cell cycle analysis of B cells from *vav*^{-/-} and wild-type mice. Small dense B cells were cultured with either monoclonal anti-IgM (10 μ g/ml b.7.6) or LPS (10 μ g/ml) for the indicated times. Cells were permeabilized and stained with PI and fluorescein isothiocyanate to measure DNA and protein content, respectively, prior to FACS analysis. Table I shows the percentages of cells in each phase of the cell cycle, determined by their protein and DNA contents.

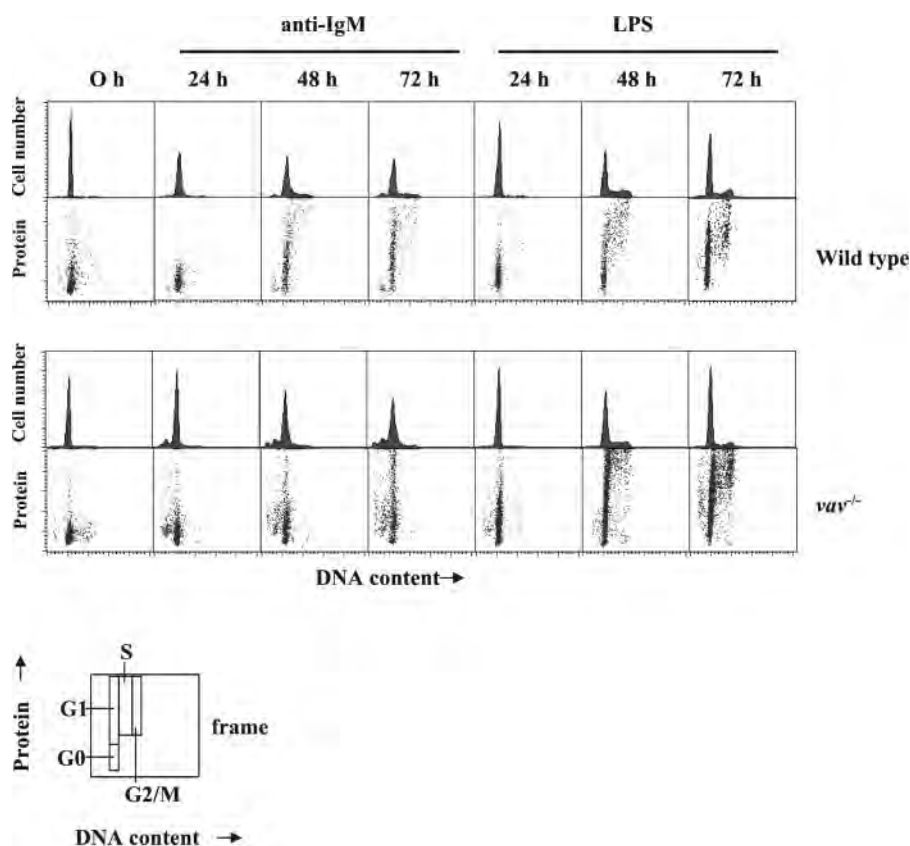


TABLE I
Fraction of *vav*^{-/-} and wild-type small dense B cells in each phase of the cell cycle

		Cells were stimulated and analysed as described in the legend to Fig. 2.				
		G ₀	G ₁	S	G ₂ /M	
Wild type	Anti-IgM	24 h	64	33	3	0
		48 h	14	61	19	6
		72 h	9	66	14	11
	LPS	24 h	35	60	2	3
		48 h	17	46	21	16
		72 h	20	52	10	18
<i>Vav</i> ^{-/-}	Anti-IgM	24 h	77	22	0	1
		48 h	52	45	2	1
		72 h	35	62	3	0
	LPS	24 h	27	65	5	3
		48 h	11	59	16	14
		72 h	6	74	9	11

These data confirm the previously reported inability of Vav-null cells to proliferate in response to antigen receptor-mediated stimuli, and extend these findings to show that this defect occurs after the cells have entered G₁ phase.

Expression of Cell Cycle Regulatory Proteins following Anti-IgM or LPS Treatment—We have shown previously that proliferative signals emanating from the B cell receptor converge on the pRB/E2F pathway, and that cyclin D2 is an essential effector of pRB phosphorylation and subsequent activation of E2F regulated genes and cell cycle entry in response to anti-IgM. Since Vav-null B cells fail to enter S phase in response to anti-IgM, and *vav*^{-/-} mice share phenotypic features with *cyclin D2*^{-/-} mice, we next sought to ascertain if a defect in cyclin D2 regulation was responsible for the inability of Vav-null B cells to proliferate in response to anti-IgM. Consistent with our hypothesis, there was no induction of cyclin D2 in

response to anti-IgM stimulation of Vav-null B cells (Fig. 3), even after 48 and 72 h, by which time cell cycle analyses demonstrated that they had entered G₁. By contrast, cyclin D2 was induced by 24 h in wild-type B cells stimulated with anti-IgM and in both wild-type and Vav-null cells treated with LPS. This induction of cyclin D2 was concomitant with a down-regulation of the cdk inhibitor, p27. Interestingly, this down-regulation of p27 also occurred in the Vav-null cells treated with anti-IgM which failed to up-regulate cyclin D2. D-type cyclins are thought to be responsible for sequestering p27 and their activation ultimately leads to its degradation in response to mitogenic stimuli (21). However, the decrease in p27 levels in this case is possibly due to general protein degradation as a result of cells undergoing apoptosis. Using antibodies that specifically recognize CDK4/6 phosphorylated residues on pRb (21, 28), we measured the *in vivo* D-type cyclin-associated CDK (4 and 6) activity in these cells. In all cases, induction of cyclin D2 was associated with a subsequent induction of CDK4/6 activity, followed by an induction of cyclin D3, the late G₁ cyclin, cyclin E, and the expression and activity of its catalytic partner, CDK2. However, in Vav-null cells treated with anti-IgM, induction of CDK4/6 activity and other downstream events did not occur. It is notable that cyclin E expression is induced faster and at a higher level in LPS-treated wild-type cells compared with those treated with anti-IgM. Since cyclin D2 accumulation is important for passage through the restriction point, while cyclin E is essential for S phase initiation (18), the earlier expression of cyclin E in LPS-treated cells compared with anti-IgM-treated cells explains the greater level of DNA synthesis observed (Fig. 1.). We have shown previously that cyclin D3 can partially compensate for cyclin D2 in *cyclin D2*^{-/-} B cells stimulated with anti-IgM. There was no induction of cyclin D3 in anti-IgM stimulated *vav*^{-/-} B cells, consistent with their inability to enter S phase and absence of CDK activity, and as previously reported (29), B cells did not express cyclin D1 (data not shown). The down-regulation in

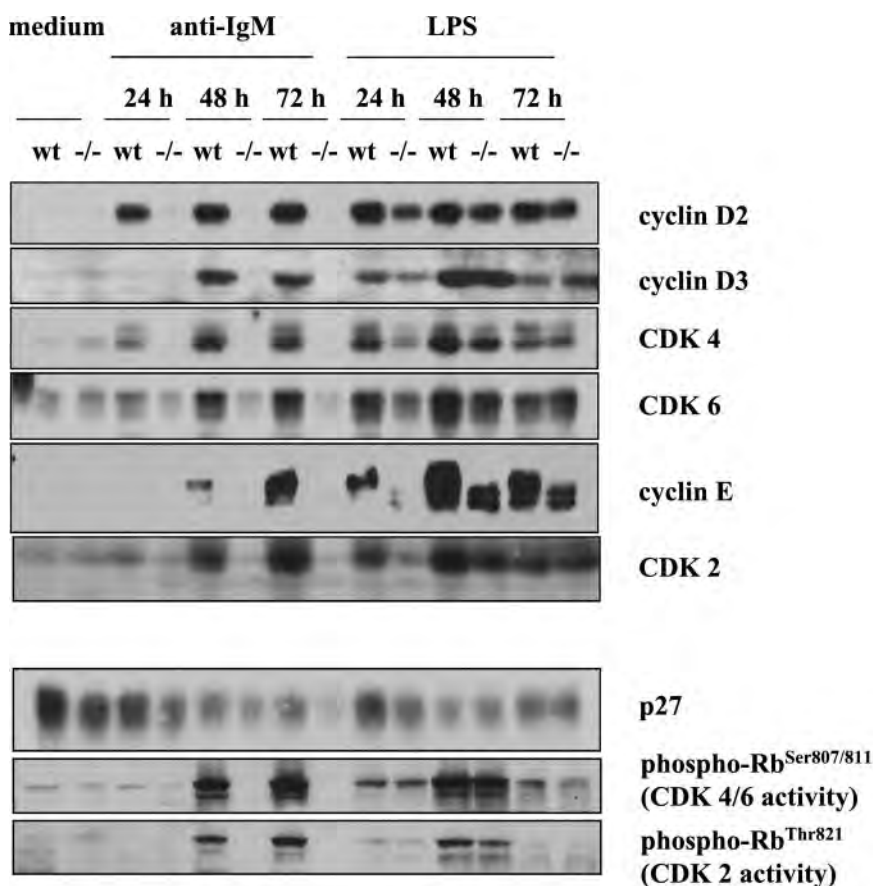


FIG. 3. **Expression of cell cycle regulatory proteins from *vav*^{-/-} and wild-type mice.** Small dense B cells were cultured with either monoclonal anti-IgM (10 μ g/ml b.7.6) or LPS (10 μ g/ml) for the indicated times. Whole cell lysates were prepared for Western blotting as described above.

CDK activity observed at 72 h in cells treated with LPS is consistent with cells having passed through S phase of the cell cycle (Figs. 1 and 2). Also of note, are the lower levels of cyclin E observed in Vav-null cells treated with LPS compared with wild-type cells (Fig. 3), but this did not appear to be rate-limiting for CDK 2 activity or S-phase induction.

Increased Cross-linking of the BCR by Anti-IgM in *vav*^{-/-} and Wild-type B Cells—It has been proposed that Vav plays an important role in setting the threshold for antigen receptor-mediated stimulation of B lymphocytes, since increased degrees of cross-linking could partially rescue the proliferative defect in the anti-IgM response of *vav*^{-/-} B cells (11). As a first step to examining the molecular basis for this response, we sought to analyze the proliferative response of small dense resting B cells in response to polyclonal anti-IgM, which has a higher degree of cross-linking to the BCR than that of the b.7.6 monoclonal anti-IgM. Previous work has demonstrated a differential proliferative response to these two stimuli in the presence of interleukin-4 (30). As has been shown previously for the total B cell population, Vav-null B cells were now able to proliferate in response to BCR cross-linking, albeit poorly in comparison to their wild-type counterparts (Fig. 4A). We next examined the expression of cell cycle regulatory proteins in these cells, and found that cyclin D2 was up-regulated in response to polyclonal anti-IgM, although its expression was lower than that observed in their wild-type counterparts. Since there was no further increase in [³H]thymidine incorporation or in cyclin D2 expression between 48 and 72 h after BCR cross-linking in Vav-null cells, it appears to be a quantitatively lower response, rather than a delayed response compared with wild-type cells. The lower level of cyclin D2 induced in *vav*^{-/-} cells is associated with a lower induction of the downstream molecules cyclin D3 and cyclin E, reduced cdk activity and a smaller reduction in p27 expression. Thus, these data further

implicate cyclin D2 as a specific target of Vav downstream of the BCR.

Expression and Activation of Downstream Signaling Pathways following BCR Stimulation of *vav*^{-/-} and Wild-type B-cells—As described previously, Vav contains several protein interaction domains in addition to its GEF activity, which suggests it may be important in several signaling pathways that might target cyclin D2 induction via the BCR. As a first step to identifying which of these pathways are involved, we analyzed the expression and activity of key enzymes by Western blotting, using antibodies raised against their phosphorylated, active forms. In addition to analyzing the rapid activation of these signaling intermediates in response to receptor cross-linking, we also looked for more prolonged activation which might be responsible for activating delayed-early genes such as cyclin D2. Vav has been shown to function as a GEF with selectivity for the Rho family GTPase, Rac1 (1). One potential downstream effector of Vav is the stress-activated kinase, p38 MAPK, which is downstream of Rac1 (31). Vav has been reported to integrate signals via the T cell receptor with p38 MAPK (32, 33), and there are several reports of p38 MAPK activation in response to BCR cross-linking (34–38). The biological consequences of p38 MAPK activation in B cells, however, remains unclear. Fig. 5 shows that p38 MAPK is rapidly activated in response to BCR cross-linking in wild-type B cells, with levels approaching the basal state after 2 h. In the Vav-null cells, however, there was no detectable p38 MAPK activity, indicating that this kinase might play a role in mediating the proliferative response to BCR cross-linking. p38 MAPK has frequently been implicated in the regulation of D-type cyclins (39–44), although none of these implicates this pathway in the positive regulation of cyclin D2. In contrast to p38 MAPK, there appeared to be a later up-regulation of another stress-activated kinase family member, SAPK/JNK, that persisted until 2 h

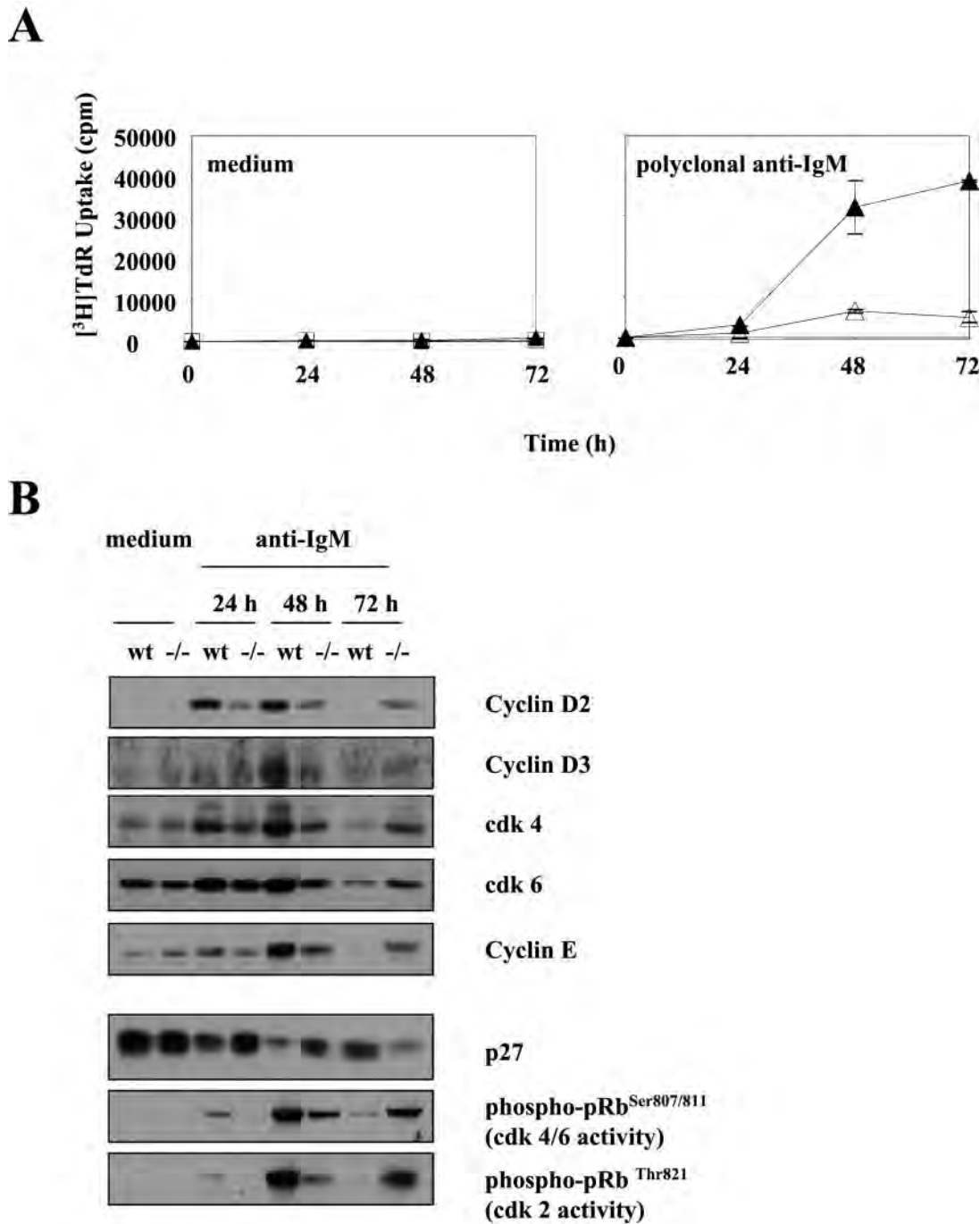
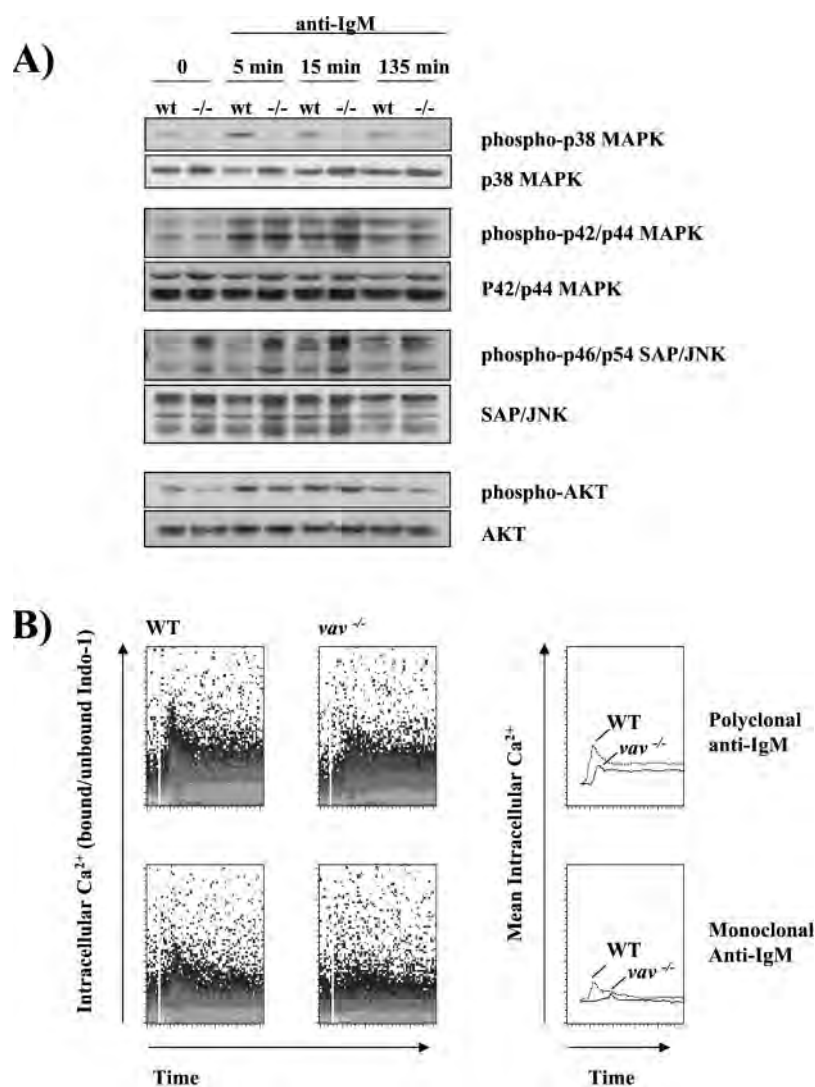


FIG. 4. Increased cross-linking of the BCR partially restores proliferation of *vav*^{-/-} B cells. **A**, [³H]thymidine incorporation was used to monitor S-phase entry in cultures of small dense B cells from *vav*^{-/-} (open squares) and wild-type mice (closed triangles) following treatment with polyclonal anti-IgM (10 μ g/ml F(ab')₂) or with no stimulation. Each data point represents a quadruplicate mean (\pm S.D.). **B**, small dense B cells were cultured with polyclonal anti-IgM (10 μ g/ml F(ab')₂) for the indicated times. Whole cell lysates were prepared for Western blotting as described above.

following stimulation. This activity was augmented in the Vav-null cells compared with their wild-type counterparts, possibly compensating for the loss of p38 MAPK in these cells. The p42/p44 (ERK) MAPK pathway is most usually associated with proliferative responses to mitogens, and has been implicated in the positive regulation of the D-type cyclins (44–49). BCR cross-linking induced a rapid activation of p42/p44 MAPK in both wild-type and *vav*^{-/-} B cells, indicating that this pathway is not dependent on Vav expression. Similarly, the activation of the AKT protein kinase which lies downstream of PI 3-K, is apparently unaffected by the loss of Vav. Recent data suggests that PI 4-phosphate (PIP) 5-kinase is a downstream target of

Vav after BCR cross-linking (4). The activity of PIP 5-kinase is essential for the biosynthesis of PIP₂ the substrate for phospholipase C which generates the [Ca²⁺]_i (intracellular calcium) regulator inositol 1,4,5-trisphosphate. Fig. 5B shows that there is a diminished [Ca²⁺]_i response observed following monoclonal anti-IgM ligation of *vav*^{-/-} B cells compared with wild-type cells, whereas increased cross-linking of the BCR with polyclonal anti-IgM partially rescues this defect. Both the size of the peak of [Ca²⁺]_i and the maintained concentrations were impaired in the Vav-null cells. Thus, the inability of *vav*^{-/-} B cells to induce cyclin D2 in response to anti-IgM could be due to defective [Ca²⁺]_i mobilization.

FIG. 5. Analysis of signaling intermediates downstream of the BCR in *vav*^{-/-} and wild-type mice. *A*, small dense B cells were cultured with monoclonal anti-IgM (10 μ g/ml b.7.6) for the indicated times. Whole cell lysates were prepared for Western blotting as described above. *B*, intracellular free calcium flux in response to BCR activation. Small dense B cells from wild-type or *vav*^{-/-} mice were cross-linked with either 10 μ g/ml b.7.6 or 10 μ g/ml polyclonal anti-IgM F(ab')₂. Calcium flux was followed for 1 min prior to stimulation, and then for a further 9 min. Data presented is representative of three separate experiments.



Is p38 MAPK Activity Required for Anti-IgM-mediated B Cell Activation?—In the previous section, we demonstrated that p38 MAPK activity was absent in Vav-null B cells stimulated with anti-IgM. We next sought to determine if this loss of enzymatic activity might be responsible for the abrogated proliferative response of these cells. Thus we used a specific inhibitor of p38 MAPK, SB 203580, to block its activation in response to anti-IgM stimulation of normal small dense B cells. DNA synthesis in these cells in the presence of the drug was diminished (Fig. 6A), indicating that p38 MAPK activity is important but not essential for anti-IgM induced B cell proliferation (or that the drug is otherwise toxic to the cells at the concentration used). However, the induction of cyclin D2 in response to anti-IgM was unaffected by the presence of the inhibitor (Fig. 6B). We have already identified cyclin D2 induction as a key target of Vav downstream of the BCR, and while the proliferative response in the presence of SB 203580 is diminished it is not abrogated. Thus we conclude that p38 MAPK is not a primary target of Vav in relation to B cell activation.

Is Intracellular Calcium Homeostasis Required for Anti-IgM-mediated B-cell Activation?—We next sought to ascertain if the diminished increase in $[Ca^{2+}]_i$ observed in Vav-null B cells stimulated with anti-IgM, compared with wild-type cells, could be responsible for their inability to up-regulate cyclin D2 and progress into S-phase. SK&F 96365 is a drug that inhibits capacitative Ca^{2+} entry, that is, specific gating of Ca^{2+} entry across the plasma membrane in response to depletion of intra-

cellular stores during calcium signaling. Thus we used this drug to inhibit the re-filling of intracellular calcium stores that is required to maintain a calcium response to extracellular stimuli. In the presence of SK&F 96365, the proliferative response of small dense B cells was completely abrogated (Fig. 7A). Thus, we conclude that $[Ca^{2+}]_i$ homeostasis is an absolute requirement for anti-IgM-mediated B cell proliferation. Analysis by Western blotting revealed that SK&F 96365 also abrogated cyclin D2 induction (Fig. 7B), indicating that the diminished $[Ca^{2+}]_i$ induction observed in Vav-null cells might be responsible for their inability to up-regulate cyclin D2 in response to anti-IgM. To further test this hypothesis, we treated small dense B cells with SK&F 96365 3 h after anti-IgM cross-linking to see if the induction of cyclin D2 could still be blocked. Using this strategy, we would maintain the normal activation of early response genes (which might also require calcium homeostasis). Also, induction of cyclin D2 is first observed after this time (Ref. 29 and data not shown). The results show that SK&F 96365 could still block cyclin D2 induction, even when added 3 h following anti-IgM. In addition, there was no DNA synthesis in these cells (data not shown). Thus we conclude that the induction of early response genes in response to the initial calcium flux is insufficient for induction of cyclin D2, and that calcium homeostasis must be maintained well beyond the time of the initial increase in $[Ca^{2+}]_i$ seen upon receptor cross-linking in order for cells to progress through the cell cycle.

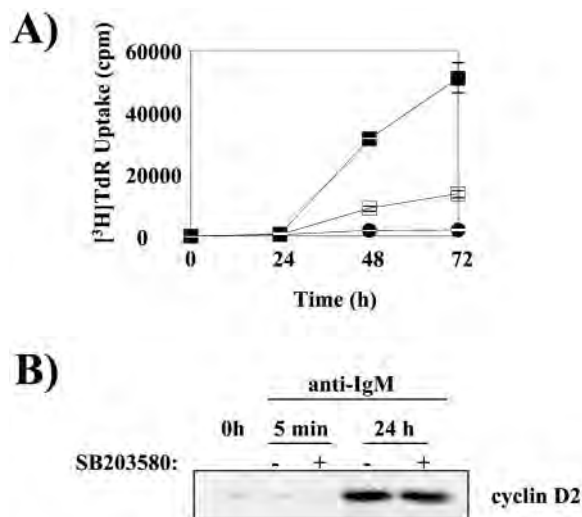


FIG. 6. Inhibition of p38 MAPK represses BCR-mediated proliferation but not cyclin D2 induction. A, [^3H]thymidine incorporation was used to monitor S-phase entry in cultures of small dense B cells following treatment with monoclonal anti-IgM (10 $\mu\text{g}/\text{ml}$ b.7.6) for the indicated times in the presence (*open squares*) or absence (*filled squares*) of 10 μM SB203580 or with no stimulation (*circles*). Each data point represents a quadruplicate mean (\pm S.D.). B, whole cell lysates from cells treated as described in A were prepared for Western blotting as described above.

DISCUSSION

In this study, we have furthered our investigations into the mechanism by which ligation of the BCR promotes cell cycle progression in B lymphocytes. We have shown previously that cyclin D2 is essential for BCR-mediated pocket protein hyperphosphorylation, regulation of E2F activity, and S-phase entry (22, 50). B cells from *vav*^{-/-} mice are defective in their ability to proliferate in response to BCR cross-linking, but are able to proliferate normally in response to other mitogens such as LPS. This phenotype is reminiscent of that observed in mice lacking the Vav-interacting proteins CD19 (12, 13) or Bruton's tyrosine kinase (14), but is also observed in mice null for the cell cycle regulatory protein, cyclin D2 (22, 23). In addition, all of these knockout mice have a depletion of CD5⁺ (B1) lymphocytes. In light of the similarities in phenotype observed in these mice, we hypothesized that cyclin D2 might be a downstream target of Vav (and CD19 and Bruton's tyrosine kinase). In order to address this, we analyzed the cell cycle entry of small dense B cells stimulated via the BCR or with LPS. D-type cyclin expression is rate-limiting for progression through the restriction point (R) of the cell cycle, which is located in mid to late G₁. Thus, the separation of small dense B cells, which are in G₀, allows the analysis of progression through G₁ in the absence of cells which may have already passed beyond this stage. We show that like "total" B cells (a mixture of unfractionated small and large cells) from *vav*^{-/-} mice, small dense B cells isolated from these mice are unable to proliferate in response to anti-IgM, while those isolated from their wild-type counterparts enter S-phase. In addition, we show that like total *vav*^{-/-} B cells, small dense *vav*^{-/-} B cells are able to proliferate normally in response to LPS. Furthermore, we were able to demonstrate that while Vav-null B cells were unable to progress beyond R and into S phase, they do enter G₁ phase. Thus the defect in BCR-mediated proliferation of *vav*^{-/-} B cells is located somewhere beyond early G₁ phase.

Analysis of the expression of cell cycle regulatory proteins in these cells revealed that, as predicted, cyclin D2 was not induced in response to BCR cross-linking of *vav*^{-/-} B cells, but was induced normally in response to LPS. These data reveal

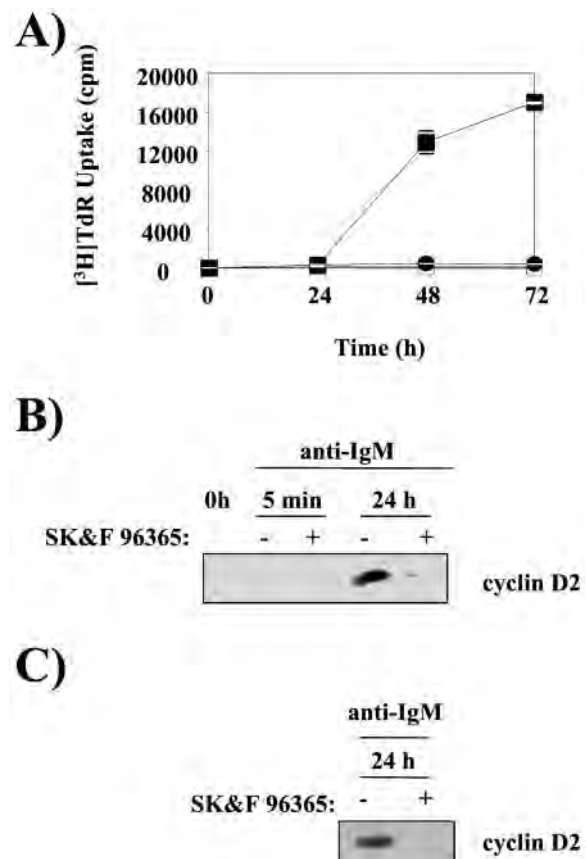


FIG. 7. Inhibition of Ca²⁺ flux abrogates BCR mediated proliferation and cyclin D2 induction. A, [^3H]thymidine incorporation was used to monitor S-phase entry in cultures of small dense B cells following treatment with monoclonal anti-IgM (10 $\mu\text{g}/\text{ml}$ b.7.6) for the indicated times in the presence (*open squares*) or absence (*filled squares*) of 10 $\mu\text{g}/\text{ml}$ SK&F 96365, or with no stimulation (*circles*). Each data point represents a quadruplicate mean (\pm S.D.). B, whole cell lysates from cells treated as described in A were prepared for Western blotting as described above. C, whole cell lysates from cultures of small dense B cells following treatment with monoclonal anti-IgM (10 $\mu\text{g}/\text{ml}$ b.7.6) for 24 h in the presence or absence of 10 $\mu\text{g}/\text{ml}$ SK&F 96365 added 3 h after the addition of anti-IgM.

the existence of a linear signaling pathway from the BCR to cyclin D2 via Vav. Previous work demonstrated the requirement for cyclin D2 in the development of CD5⁺ (B1) cells and for IgG class switching in addition to BCR-induced proliferation (22, 23). This raises the possibility that the defects in CD5⁺ (B1) cells and IgG class switching observed in Vav-null B cells (30) may be due to their inability to induce cyclin D2. The importance of this pathway in mediating proliferation of B cells in response to BCR cross-linking was further demonstrated by using a polyclonal anti-IgM to stimulate the BCR. Vav has been postulated to act as an amplifier of signals downstream of BCR ligation, since its absence can be partially compensated for by increased cross-linking of the BCR with a polyclonal anti-IgM (11, 30). Thus, Vav-null B cells stimulated with polyclonal anti-IgM were able to proliferate, but not as well as wild-type cells. The reason for this appears to be the induction of low levels of cyclin D2 in response to polyclonal anti-IgM in *vav*^{-/-} cells, resulting in a later induction of CDK4/6 activity which leads to pocket-protein hyperphosphorylation and a later induction of E2F responsive genes such as *cyclin E*. Whether or not this is due to functional compensation by another member of the Vav family (Vav 2 and Vav 3 are expressed at low levels in hemopoietic cells (2), or another mechanism remains to be determined. It has been shown recently, however, that some functional compensation does occur by Vav-2 for Vav in terms

of B cell development (51, 52). In addition, mice null for both Vav and Vav-2 are unable to respond even to increased cross-linking of the BCR. However, the proliferative response of *vav*^{-/-} B cells is still compromised in comparison to wild-type cells, emphasizing the importance of Vav in mediating the induction of sufficient amounts of rate-limiting cyclin D2 protein in response to BCR ligation. Interestingly, it was reported recently that mice null for the BLNK (B cell linker) adapter protein also have a similar phenotype to Vav-null mice (53). BLNK (also known as SLP-65 or BASH) is an SH2/3 domain containing protein that is phosphorylated by the Syk tyrosine kinase on BCR cross-linking and is then thought to recruit Bruton's tyrosine kinase, phospholipase C (PLC)- γ 2, Vav, Grb2, and Nck to integrate downstream signaling pathways. BLNK^{-/-} B cells fail to proliferate in response to BCR ligation, while responding normally to LPS, and have a depletion of CD5⁺ (B1) lymphocytes. Furthermore, they were also unable to induce cyclin D2 in response to BCR stimulation, but it is not known if, like *vav*^{-/-} B cells, they progress into early G₁.

Since Vav contains a number of protein interaction domains in addition to its GEF activity, there are numerous possible pathways in which it might promote the induction of cyclin D2 by the BCR. As a first step in establishing which of these pathways might be involved, we measured the activation of potential downstream signaling intermediates in both wild-type and *vav*^{-/-} B cells in response to anti-IgM. The most striking observation from this analysis was the absence of detectable amounts of active p38 MAPK in Vav-null B cells, while in wild-type cells the active form of this enzyme was present in resting cells, and its activity was further augmented by BCR cross-linking. This is consistent with the idea that p38 MAPK is downstream of Vav, probably due to its ability to activate Rac1 (thought to be the main target of Vav in lymphocytes), which in turn activates a mitogen-activated protein kinase cascade leading to p38 MAPK activation. However, inhibition of the activity of p38 MAPK in normal small dense B cells did not diminish the induction of cyclin D2 by BCR cross-linking, suggesting that p38 MAPK activity is not essential for this process. This is despite the decreased proliferative response of these cells, perhaps indicating that p38 targets something downstream of cyclin D2. In support of this, BLNK^{-/-} B cells which also fail to up-regulate cyclin D2, have no defect in p38 MAPK activity (53). Inhibition of p38 MAPK did, however, lead to a reduction in the proliferative capacity of these cells in response to BCR ligation. This effect may be due to the requirement for p38 MAPK activity in other parts of the cell cycle or in cellular metabolism, or could be due to nonspecific effects on other cellular enzymes/processes. We show that anti-IgM fails to activate p38 in the *vav*^{-/-} B cells, but the p38 inhibitor inhibits anti-IgM stimulated B cell proliferation without affecting cyclin D2 expression. These observations suggest that the proliferative defects in the *vav*^{-/-} B cells involve more than the failure to induce cyclin D2 expression. Nevertheless, our recent publications have shown that cyclin D2 is essential for the proliferation of B cells induced by BCR cross-linking (22, 23).

In contrast to p38 MAPK, the activity of another stress-activated kinase family member, SAPK/JNK was augmented in the absence of Vav, perhaps due to functional compensation by another Vav family member with specificity to SAPK/JNK rather than p38 MAPK. It is possible that the increased level of SAPK/JNK activity is responsible for suppressing the induction of cyclin D2 in *vav*^{-/-} B cells, but this remains to be tested. The MAPK pathway most usually associated with proliferation and the induction of cyclins is that of the p42/p44 MAPK (ERK); this enzyme, however, appeared to be activated

normally in response to anti-IgM in the absence of Vav. This is in contrast to the defective induction of p42/p44 MAPK observed in T cells stimulated via the TCR (54). We additionally show that the activation of the Akt protein kinase (a downstream target of PI 3K) is preserved in the absence of Vav. The pleckstrin homology domain of Vav binds to the lipid products of the reaction catalyzed by the lipid kinase activity of PI 3K, promoting its activation, and possibly targeting it to sites of activation within the cell (55). It is therefore possible that the inability of *vav*^{-/-} B cells to up-regulate cyclin D2 in response to BCR ligation was due to negative feedback to PI 3K activity. This is an important consideration, since PI 3K (and Akt) has been frequently implicated in promoting cell cycle progression, and D-type cyclin induction (56). The maintenance of Akt activation in the absence of Vav, however, suggests that down-regulation of PI 3K activity via negative feedback is probably not the mechanism by which cyclin D2 induction is negated in *vav*^{-/-} B cells.

It has been demonstrated previously that *vav*^{-/-} B cells have defects in calcium homeostasis following engagement of antigen receptors and CD19 (4). However, calcium mobilization was demonstrated to be normal in response to BCR ligation alone (4, 51). Upon BCR engagement, PI 4-phosphate (PIP) 5-kinase phosphorylates PIP to generate PI 4,5-bisphosphate (PIP₂), the substrate for PLC. PLC γ 2 hydrolyses PIP₂ to form inositol 1,4,5-trisphosphate and diacylglycerols, which regulate intracellular calcium and PKC, respectively. We demonstrate here, that small dense *vav*^{-/-} B cells stimulated with monoclonal anti-IgM do not maintain calcium fluxes as well as their wild-type counterparts. Increased cross-linking of the BCR with polyclonal anti-IgM results in a calcium flux which, while still lower than that observed in wild-type cells, is greater than that achieved with monoclonal anti-IgM. The apparent discrepancy with reports that calcium mobilization is normal in response to BCR cross-linking of Vav-null cells is possibly due in part to the increased cross-linking of the receptor used in these studies (either with higher concentrations of monoclonal anti-IgM (51) or with polyclonal anti-IgM (4, 6, 7) and may also be due in part to the different populations of B cells used. None of these studies used small dense B cells, which may differ in their response to large cells that are already in G₁ phase of the cell cycle. Since increased cross-linking of the BCR can also partially rescue the proliferative defect of Vav-null B cells by partially restoring cyclin D2 induction, we hypothesized that the inability to maintain a [Ca²⁺]_i flux might be the reason that small dense *vav*^{-/-} B cells are unable to respond adequately to BCR cross-linking. By treating normal small dense B cells with an inhibitor of capacitative Ca²⁺ entry we were able to show that maintenance of intracellular calcium is necessary for cyclin D2 induction. Since we have already demonstrated that an inability of *vav*^{-/-} B cells to induce cyclin D2 following BCR ligation is central to their inability to proliferate in response to this stimulus, we speculate that the role of Vav in regulating intracellular calcium might be central to its regulation of cyclin D2 in response to BCR engagement. In addition, we chose to inhibit capacitative Ca²⁺ entry several hours following BCR engagement, and were still able to block the induction of cyclin D2. These data show that maintenance of intracellular calcium is not only required for events proximal to BCR ligation, but also for the generation of downstream events. More sophisticated analyses and manipulation of intracellular calcium will be required to determine the precise requirements for calcium homeostasis in relation to cyclin D2 regulation in both normal and *vav*^{-/-} B cells, and to assess the potential involvement of calcium dependent factors. In support of the potential role of calcium signaling in BCR-mediated cyclin D2 induction is the

fact that LPS induced B cell activation (which is normal in Vav-null B cells) normally occurs by a calcium-independent mechanism (57). Further studies are required to determine if there is a general requirement of capacitative Ca^{2+} entry for D-type cyclin induction in other cell systems. Numerous studies have implicated the requirement for Ca^{2+} in proliferation (58), and a recent report demonstrates the necessity to maintain intracellular calcium for timely progression through G_1/S (59). Confirmation of the importance of the Vav family in mobilizing intracellular calcium has been shown recently, in that mice null for both Vav and Vav-2 were unable to generate any calcium response following BCR ligation (even with increased cross-linking), which was attributed to be the cause of their complete inability to proliferate following these stimuli (51, 52).

Further studies into the mechanism of regulation of cyclin D2 by Vav should be informative in further defining the pathway linking cyclin D2 and subsequent cell cycle progression with BCR engagement, while delineation of this pathway may reveal potential therapeutic strategies for immunological disorders. Vav was first isolated by its transforming ability, and has been shown to be constitutively activated by the BCR-ABL oncogene (60), which is responsible for the pathogenesis of chronic myelogenous leukemia and Philadelphia 1 (Ph1) acute lymphoblastic leukemia. Furthermore, we have shown recently that BCR-ABL promotes hemopoietic cell proliferation through induction of cyclin D2 (61) (and down-regulation of p27), raising the possibility that it does so via Vav.

Acknowledgment—We thank N. Shaun B. Thomas for helpful discussion and critical review of the manuscript.

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**MECHANISMS OF SIGNAL
TRANSDUCTION:**

**Vav Is Required for Cyclin D2 Induction
and Proliferation of Mouse B Lymphocytes
Activated via the Antigen Receptor**

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J. Biol. Chem. 2001, 276:41040-41048.

doi: 10.1074/jbc.M105305200 originally published online September 6, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M105305200](https://doi.org/10.1074/jbc.M105305200)

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