The Journal of Immunology

CD38 Signaling Regulates B Lymphocyte Activation via a Phospholipase C (PLC)- γ 2-Independent, Protein Kinase C, Phosphatidylcholine-PLC, and Phospholipase D-Dependent Signaling Cascade

Miguel E. Moreno-García, Lucia N. López-Bojórques, Alejandro Zentella, Lisa A. Humphries, David J. Rawlings and Leopoldo Santos-Argumedo

J Immunol 2005; 174:2687-2695; ;
doi: 10.4049/jimmunol.174.5.2687
http://www.jimmunol.org/content/174/5/2687

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CD38 Signaling Regulates B Lymphocyte Activation via a Phospholipase C (PLC)-γ2-Independent, Protein Kinase C, Phosphatidylcholine-PLC, and Phospholipase D-Dependent Signaling Cascade[1]

Miguel E. Moreno-García,*† Lucia N. López-Bojórques,‡ Alejandro Zentella,‡ Lisa A. Humphries,§ David J. Rawlings,§ and Leopoldo Santos-Argumedo[2†]

The CD38 cell surface receptor is a potent activator for splenic, B lymphocytes. The molecular mechanisms regulating this response, however, remain incompletely characterized. Activation of the nonreceptor tyrosine kinase, Btk, is essential for CD38 downstream signaling function. The major Btk-dependent substrate in B cells, phospholipase C-γ2 (PLC-γ2), functions to generate the key secondary messengers, inositol-1,4,5 trisphosphate and diacylglycerol. Surprisingly, CD38 ligation results in no detectable increase in phosphoinositide metabolism and only a minimal increase in cytosolic calcium. We hypothesized that Btk functioned independently of PLC-γ2 in the CD38 signaling pathway. Accordingly, we demonstrate that CD38 cross-linking does not result in the functional phosphorylation of PLC-γ2 nor an increase in inositol-1,4,5 trisphosphate production. Furthermore, splenic B cells exhibit a normal CD38-mediated, proliferative response in the presence of the phosphoinositide-PLC inhibitor, U73122. Conversely, protein kinase C (PKC) β-deficient mice, or PKC inhibitors, indicated the requirement for diacylglycerol-dependent PKC isoforms in this pathway. Loss of PKC activity blocked CD38-dependent, B cell proliferation, NF-κB activation, and subsequent expression of cyclin-D2. These results suggested that an alternate diacylglycerol-producing phospholipase must participate in CD38 signaling. Consistent with this idea, CD38 increased the enzymatic activity of the phosphatidylcholine (PC)-metabolizing enzymes, PC-PLC and phospholipase D. The PC-PLC inhibitor, D609, completely blocked CD38-dependent B cell proliferation, IκB-α degradation, and cyclin-D2 expression. Analysis of Btk mutant B cells demonstrated a partial requirement for Btk in the activation of both enzymes. Taken together, these data demonstrate that CD38 initiates a novel signaling cascade leading to Btk-, PC-PLC-, and phospholipase D-dependent, PLC-γ2-independent, B lymphocyte activation. ***The Journal of Immunology,*** **2005, 174: 2687–2695.**

The CD38 is a 42-kDa type II transmembrane glycoprotein expressed in many tissues, including most hemopoietic cell lineages (1–3). CD38 was initially identified as an ectoenzyme able to hydrolyze β-nicotinamide adenine dinucleotide to cyclic adenosine-diphosphoribose, adenosine diphosphoribose, and nicotinamide (4, 5). It is known that cyclic adenosine-diphosphoribose triggers calcium mobilization from the endoplasmic reticulum (ER)[3] through ryanodine receptors (6). CD38, however, also displays activity as a cell surface receptor. Activation through CD38 with agonistic Abs triggers a variety of responses including: cell proliferation, rescue from apoptosis, inducible protein tyrosine phosphorylation, a low level increase in intracellular calcium, and the expression of several surface molecules including MHC-II and CD69 (7–9).

Although preliminary investigations of the CD38 receptor have revealed varied functions, the signaling events mediated by CD38 engagement remain unclear. Indeed, while the proliferative response of mature splenic B cells to CD38 has been shown to require both Src (Lyn and Fyn) (10) and Tec family kinases (Btk) (11, 12), their downstream substrates remain unknown. A well-characterized target of Btk in BCR-mediated signaling is the phosphoinositide-phospholipase C-γ2 (PI-PLC-γ2). Btk is essential for the activation of PLC-γ2 (13–15), and these enzymes work in concert to produce downstream signals required for B cell survival, differentiation, and proliferation. Once activated, PLC-γ2 mediates hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the sustained production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) required for an initial intracellular calcium release and protein kinase C (PKC) activation, respectively (16).

Previous studies, however, have suggested that CD38-mediated intracellular calcium flux is dependent on extracellular calcium sources and occurs by a mechanism that is independent of inositol

*Departments of Cell Biology and †Molecular Biomedicine, Centro de Investigación y Estudios Avanzados, Mexico D.F. Mexico; ‡Department of Cell Biology, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico D.F., Mexico; and §Department of Pediatrics, University of Washington School of Medicine, Seattle, WA 98195

Received for publication December 19, 2003. Accepted for publication December 13, 2004.

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

[1] This work was supported by grants to L.S.-A. from Consejo Nacional de Ciencia y Tecnología (Conacyt), México (28093N and 33497N). D.J.R. is the recipient of a McDonnell Scholar Award, a Leukemia and Lymphoma Society Scholar Award, and the Joan J. Drake Grant for Excellence in Cancer Research. This work was partially supported by National Institutes of Health Grants HD37091, CA81140, AI38348, and AI33617, and the American Cancer Society.

[2] Address correspondence and reprint requests to Dr. Leopoldo Santos-Argumedo, Departamento de Biomedicina Molecular, Centro de Investigación y Estudios Avanzados del I.P.N., Apartado Postal 14-740, cp 07360, México D.F. México. E-mail address: lesantos@cinvestav.mx

[3] Abbreviations used in this paper: ER, endoplasmic reticulum; DAG, diacylglycerol; IP_3, inositol-1,4,5 trisphosphate; PC, phosphatidylcholine; PI-PLC, phosphoinositide-phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PY, phosphotyrosine; WT, wild type.

0022-1767/05/$02.00

phosphate generation and internal calcium-store release (8, 9). Although these observations suggest that PLC-γ2 function may not be required for CD38 signaling, they are seemingly in conflict with the requirement of Btk in CD38 signaling.

To begin to address this apparent paradox, we evaluated the participation of PLC-γ2 in CD38 signaling in murine splenic B cells. Our studies show that CD38-dependent proliferation is unaffected by the addition of a PLC-specific inhibitor (U73122) and that cross-linking of the CD38 receptor fails to induce IP_3 generation. Consistent with these results, there was a loss of receptor-dependent, functional PLC-γ2 phosphorylation, as evidenced by the absence of phosphorylation at PLC-γ2 Y759, a key regulatory site (17–19). Together these data indicated that PLC-γ2 was not required for the CD38 signaling pathway. Conversely, B cells deficient in the major classical PKC isoform, PKC-βI and -βII, exhibited a 2-fold reduction in the proliferative response to CD38, and the addition of Ro-318425, a potent inhibitor of classical and novel PKC isoforms, completely abrogated the CD38-induced proliferative response of normal B lymphocytes. Furthermore, CD38 engagement led to PKC-dependent degradation of IκB-α and translocation of NF-κB into the nucleus, resulting in the production of the NF-κB-dependent target, cyclin-D2.

Interestingly, it has been shown that both phosphatidylcholine-specific PLC and phospholipase D (PC-PLC and PLD) are able to produce the DAG necessary for PKC activation in several signaling receptors without concomitant production of IP_3 (20–23). Indeed, we found that CD38 activated both the PC-PLC and PLD, and the addition of a specific PC-PLC inhibitor (D609) blocked CD38-induced proliferation, IκB-α degradation, and cyclin-D2 synthesis. These data support a model in which CD38-dependent proliferation occurs via a novel Btk/PC-PLC, PLD/PKC/NF-κB pathway, independent of PLC-γ2.

Materials and Methods

Cell preparation

Female BALB/c, BALB/c-XID, PKC-βI and -$\beta II^{-/-}$, and $PKC^{+/-}$ (24) mice were used at 6–8 wk of age for all experiments. For splenic B cell purification, T cells were eliminated by two rounds of panning using anti-Thy-1 Abs.

Inhibitors

Ro-318425, an inhibitor of the PKC α, βI-II, γ, and ε isoforms, was used at 62 nM; the PC-PLC inhibitor, D609, was used at 12, 13.5, and 15 μg/ml. The PI-PLC inhibitor, U73122, and its inactive analog, U73343, were used at 0.3 μM. All of the inhibitors were purchased from Calbiochem. Depending on the experiment, cells were pretreated for 30 min and throughout the experiment with inhibitors.

Proliferation assays

For proliferation assays, splenic B cells (2×10^5 cells/well) were incubated 48 h with the following stimuli: 50 μg/ml with the mAb anti-CD38 (NIM-R5) (9); 10 μg/ml anti-IgM $F(ab')_2$ (Jackson ImmunoResearch Laboratories); 20 μg of LPS from *Escherichia coli* serotype O55:B5 (Sigma-Aldrich); anti-CD40 (IC10 supernatant diluted 1/4) (25); all the stimuli were performed in the presence of murine rIL-4 at 20 U/ml (Genzyme). The cells were pulsed with 1 μCi of [^{3}H]thymidine (NEN) 12 h before harvesting, and thymidine incorporation was measured in a beta scintillation counter (PerkinElmer Wallac).

Flow cytometry

Purified splenocytes (10^6) were incubated 30 min with a mixture of B220-FITC (BD Pharmingen) plus NIM-R5-PE (Southern Biotechnology Associates)-labeled Abs. Cell suspensions were analyzed using a FACSCalibur cytometer (BD Biosciences).

Immunoprecipitation and Western blot

For PLC-γ2 immunoprecipitation, 5×10^7 B cells were stimulated with 50 μg/ml NIM-R5; 10 μg/ml anti-IgM $F(ab')_2$; or 50 μg/ml nonspecific rat IgG for 0.5, 1, 5, and 10 min. Cells were lysed in buffer containing 150 mM NaCl, 10 mM Tris-base, pH 7.2, 2 mM Na_3VO_4, 0.4 mM EDTA, 10 mM NaF, 1 mM PMSF, 5 μg/ml aprotinin and leupeptin, and 1% Nonidet P-40. PLC-γ2 was immunoprecipitated with an anti-PLC-γ2 Ab (Santa Cruz Biotechnology) for 1 h at 4°C, and Ag-Ab complexes were precipitated using protein G-Sepharose (Zymed Laboratories). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. For phosphotyrosine (PY) detection, the membrane was probed with the anti-phosphotyrosine Ab 4G10-HRP (Upstate Biotechnology) and developed by ECL-Plus (Amersham Biosciences). The membrane was stripped and reanalyzed with the anti-PLC-γ2 Ab, followed by an anti-rabbit-HRP Ab (DakoCytomation). For the detection of the phosphorylated tyrosine residue 759 of PLC-γ2 (pY759), B cells were stimulated for 1, 5, and 10 min with anti-CD38, anti-IgM, and rat IgG, and pY759 was detected by Western blot from total lysates using an anti-pY759 phosphospecific rabbit polyclonal Ab (19), followed by an anti-rabbit-HRP Ab. For IκB-α degradation assays, cells (10^7 per stimuli) were preincubated for 30 min with 50 μM cycloheximide and stimulated for 30, 60, or 120 min with anti-CD38, anti-IgM, or nonspecific rat IgG, at the concentrations described above. LPS at 20 μg/ml and 2 μg/ml PMA (Sigma-Aldrich) were used as controls. Cell lysates were prepared, and 20 μg of total proteins was used for immunoblotting. IkB-α was detected using a polyclonal Ab anti-IκB-α (Santa Cruz Biotechnology), followed by an anti-rabbit-HRP Ab (DakoCytomation).

EMSA

B cells were activated with rat IgG, anti-CD38, anti-IgM, and LPS at the concentrations described above. Nuclear extracts were obtained, as described (26), and 10 μg of nuclear extracts/sample was incubated with a ^{32}P-labeled oligonucleotide containing the κB consensus sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Mobility shift was analyzed on a nondenaturing 7.5% polyacrylamide gel, as previously described (27). Bands were developed by autoradiography in phosphorous plates using a digitalizing system (Storm; Amersham Biosciences) and analyzed with the ImageQuant software (Amersham Biosciences).

Cyclin-D2 expression

Splenic B cells were cultured in six-well tissue culture plates at a density of 1×10^7 cells/ml for 12 h with anti-CD38, anti-IgM, rat IgG, and LPS at the concentrations mentioned above in the presence of 20 U/ml IL-4. Cells were harvested, pelleted at 500 × *g*, and lysed in 100 μl of lysis buffer. Proteins were resolved by SDS-PAGE (12%), and cyclin-D2 expression was detected by Western blot using a polyclonal Ab to cyclin-D2 (Santa Cruz Biotechnology).

IP_3 production, PC-PLC, and PLD activation analysis

Purified B cells (1×10^8 cells/500 μl) were stimulated with anti-CD38 (100 μg/ml), rat IgG (100 μg/ml), or anti-IgM (60 μg/ml) for 0, 1, and 2.5 min, and IP_3 was purified and measured following the manufacturer instructions using an inositol-1,4,5-trisphosphate ^{3}H Radioreceptor Assay kit (PerkinElmer; catalogue NEK064). To analyze the activation of PC-PLC and PLD, the cells were stimulated for 0, 1, 30, 60, 120, and 180 min and lysed, as previously described. Protein lysates (15 μg/sample) were analyzed for PC-PLC activity using an Amplex Red PC-PLC assay kit, according to the manufacturer instruction (catalogue A-12218; Molecular Probes). In this enzyme-coupled assay, PC-PLC activity is monitored indirectly using 10-acetyl-3,7-dihydrophenoxacine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. First, PC-PLC converts exogenous PC (lecithin) substrate to phosphocholine and DAG. Phosphocholine is hydrolyzed to choline by alkaline phosphatase. Choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of HRP reacts with Amplex Red in a 1:1 stoichiometry to generate resorufin, which is excited at 540 nm and detected at 590 nm using a microplate fluorometer (Fluoromark; Bio-Rad). For PLD assays, a similar protocol was used, but alkaline phosphatase was excluded from the system (as recommended by the manufacturer). Thus, choline and not phosphocholine is quantified with this assay (catalogue A-12219; Molecular Probes).

Results

CD38-mediated B lymphocyte proliferation occurs independently of PLC-γ2 activation

Btk has been shown to be crucial for CD38-induced B lymphocyte proliferation (11, 12). As PLC-γ2 is a major substrate for Btk in BCR-mediated signaling (13–15), we evaluated the requirement for PLC-γ2 in CD38-mediated murine splenocyte activation (Fig.

1). Initial analysis of whole cell lysates revealed the inducible increase in global tyrosine PY of cellular substrates peaked at ~5 min following stimulation with a CD38 agonist (data not shown). This time point was therefore used to further evaluate total, and activation site-specific, PLC-γ2 tyrosine phosphorylation. Using a pan-phosphotyrosine-specific Ab, immunoprecipitated PLC-γ2 was found to be rapidly phosphorylated following CD38 cross-linking. The relative levels of PLC-γ2 total phosphorylation induced by anti-CD38 or anti-IgM engagement were indistinguishable (Fig. 1*A*), suggesting that CD38 stimulation may lead to the activation of PLC-γ2. In contrast, while addition of the PI-PLC inhibitor, U73122, markedly reduced BCR-dependent B cell proliferation, addition of this drug had no effect on CD38-dependent proliferation. CD38-dependent proliferation was comparable in untreated control cells, cells treated with the inactive analog U73343, and cells treated with U73122 (Fig. 1*B*). U73122 also had no appreciable effect on LPS-dependent proliferation, a surrogate PLC-γ2-independent pathway (28–30). These results suggested a dispensable role for PLC-γ2 in CD38-mediated proliferation.

To more precisely characterize the extent of PLC-γ2 activation, we next examined PLC-γ2 site-specific phosphorylation. Recent reports have shown that activation of PLC-γ2 is dependent on the phosphorylation of specific tyrosine residues, including Y753, Y759, Y1197, and Y1217, and that mutation of these residues impairs the biological function of the enzyme (17, 18). Furthermore, recent data also demonstrate that Btk/Tec kinases are uniquely and specifically required for site-specific phosphorylation of Y753 and Y759 within the Src homology 2-Src homology 3 linker region of PLC-γ2 (19). Thus, direct assessment of Y759 site-specific phosphorylation is a more accurate measure of PLC-γ2 activation in comparison with evaluation using global phosphotyrosine Abs. Fig. 2*A* clearly shows that BCR engagement leads to a rapid, prominent increase in tyrosine phosphorylation at the PLC-γ2 regulatory residue, Y759. In contrast, Y759 site-specific phosphorylation was not observed in CD38-stimulated cells at any time point following receptor engagement (Fig. 2*A*). The global increase in the phosphotyrosine content of PLC-γ2 (observed in response to CD38 cross-linking) most likely reflects phosphorylation of alternative, Btk-independent sites (including Y1197 and Y1217) similar to that seen in BCR-stimulated, Btk-deficient B cells (19). Consistent with these results, engagement of CD38 did not induce IP_3 production, while IgM stimulation induced a rapid and prominent increase in the production of this key second messenger (Fig. 2*B*). These results demonstrate that CD38 engagement leads to the incomplete, nonfunctional, tyrosine phosphorylation of PLCγ2, and that these events correlate directly with a lack of IP_3 generation. Together, these findings strongly suggest that PLC-γ2 is not required for CD38 signaling.

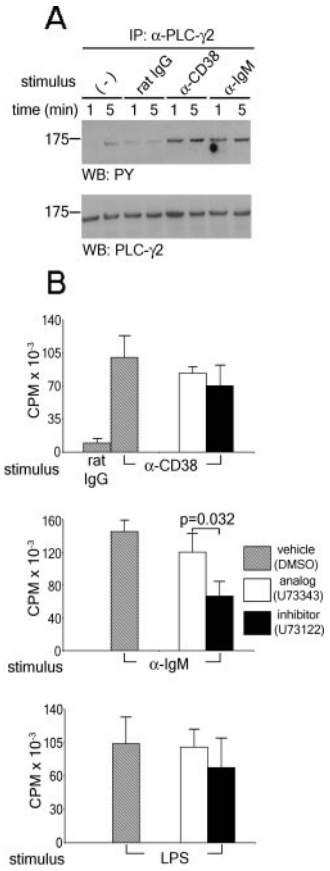

FIGURE 1. PLC-γ2 is tyrosine phosphorylated after CD38 cross-linking, but does not participate in the CD38-induced proliferation of B cells. *A*, Splenic B cells were stimulated with anti-CD38, anti-IgM F(ab′)$_2$, or rat IgG Abs for the times indicated in the figure, and PLC-γ2 was immunoprecipitated with an anti-PLC-γ2 Ab. PY detection and protein loading analysis were done with 4G10-HRP Ab (*upper panel*) and with anti-PLC-γ2 Ab (*lower panel*), respectively. *B*, B cells were preincubated with vehicle (DMSO, ▨) or with 0.3 μM PI-PLC inhibitor U73122 (■) or its inactive analog U73343 (□), followed by the activation of the cells (in the presence of 20 U/ml IL-4) with rat IgG, anti-CD38, anti-IgM F(ab′)$_2$, or LPS, and the proliferation was measured by [^{3}H]thymidine incorporation. Higher U73122 concentrations (0.6 μM or higher) were experimentally excluded as they nonspecifically blocked the LPS-dependent proliferation. The groups were compared with unpaired Student's *t* test using the program INSTAT 2.02 (GraphPad), in which *p* values ≤0.05 were considered to be significant.

DAG-dependent PKC isoforms are indispensable for CD38-mediated B cell proliferation

Previous reports have shown that PKC may be involved in CD38 signaling events in T lymphocytes (31). In addition, recent studies

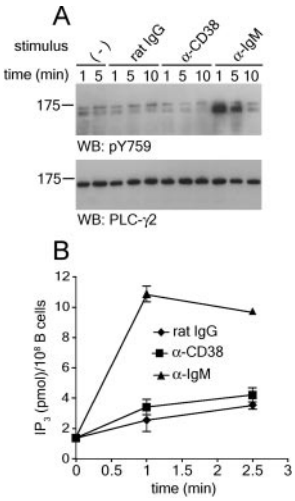

FIGURE 2. CD38 is able to induce neither IP_3 production nor phosphorylation of tyrosine-759 (pY759) of PLC-γ2. *A*, Splenic B cells (1×10^7) were incubated during the times indicated in the figure with rat IgG, anti-CD38, and anti-IgM F(ab′)$_2$ Abs. Total cell lysates were prepared, and the pY759 was detected by Western blot using a rabbit polyclonal Ab against the PY-759 of PLC-γ2. *B*, B lymphocytes (1×10^8 cells/500 ml) were stimulated with rat IgG, anti-CD38, and anti-IgM F(ab′)$_2$ Abs, and then IP_3 production was quantified by an inositol-1,4,5-trisphosphate ^{3}H Radioreceptor Assay kit (PerkinElmer), as detailed in *Materials and Methods*. Triangles, anti-IgM F(ab′)$_2$; squares, anti-CD38; diamonds, rat IgG.

indicate that PKC is involved in the activation of NF-κB in splenic B cells stimulated through CD38 (32). The failure to activate PLC-γ2 in CD38-stimulated B cells suggested that DAG production and subsequent PKC isoform activation (33) were either deficient, or alternatively, could occur via a PLC-γ2-independent pathway. To begin to examine the former possibility, we evaluated CD38-mediated proliferation of B lymphocytes from mice deficient in the DAG- and Ca^{2+}-dependent PKC isoforms (24) PKC-βI and -βII (PKC-$\beta^{-/-}$). The proliferative response of CD38-stimulated PKC-$\beta^{-/-}$ B lymphocytes was reduced by ~50% compared with that of PKC-$\beta^{+/-}$ cells (Fig. 3*A*). As previously characterized (24), a similar decrease in proliferation was observed in response to IgM engagement in PKC-$\beta^{-/-}$ vs PKC-$\beta^{+/-}$ B lymphocytes. In contrast, LPS-induced proliferation was similar for PKC-$\beta^{-/-}$ and PKC-$\beta^{+/-}$ splenocytes. Of note, no significant differences were observed in the percentage of $CD38^+$ cells (or in the CD38 mean fluorescence intensity) in $B220^+$ splenic B cells of PKC-$\beta^{-/-}$ vs PKC-$\beta^{+/-}$ mice. This finding indicates that the defective proliferative response in PKC-$\beta^{-/-}$ mice was not secondary to altered CD38 expression (Fig. 3*B*).

The residual proliferative activity of PKC-$\beta^{-/-}$ B cells suggested that alternative PKC isoforms were most likely also involved in the CD38 signaling pathway. To evaluate this hypothesis, primary splenic B cells were treated with the global PKC inhibitor Ro-318425 (a potent inhibitor of classical and novel PKC isoforms including PKC α, βI, II, γ, and ε) before activation by anti-CD38 or alternative stimuli (Fig. 3*C*). Ro-318425 completely blocked the proliferation induced by both CD38 and IgM. In contrast, this inhibitor had no effect on LPS- or anti-CD40-dependent B cell proliferation (34, 35). Together, these data support the conclusion that DAG-dependent PKC isoforms, including PKC-β isoforms, are required for CD38-mediated signal transduction and proliferation.

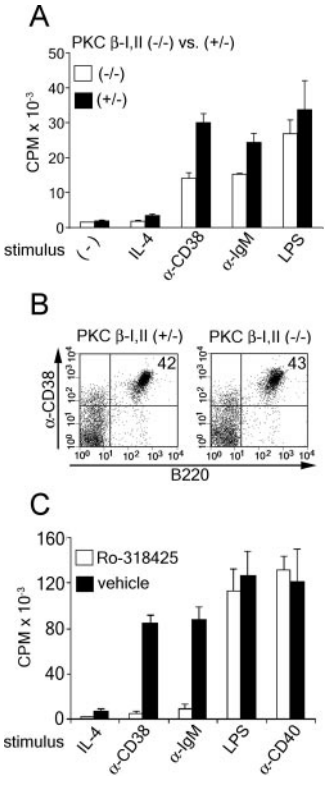

FIGURE 3. CD38 requires DAG-dependent PKC isoforms for B cell proliferation. *A*, Proliferative responses of splenic B cells stimulated (in the presence of 20 U/ml IL-4) with anti-CD38, anti-IgM $F(ab')_2$, or LPS. Proliferation was measured by [^{3}H]thymidine incorporation. Results on activation of PKC-$\beta^{-/-}$ (□) or PKC-$\beta^{+/-}$ (■) splenocytes are shown. *B*, Expression of surface CD38 on splenic B cells derived from PKC-βI and -βII$^{-/-}$ (*right*) and PKC-$\beta^{+/-}$ (*left*) mice. Numbers in the *upper right quadrants* represent the percentages of $CD38^+/B220^+$ cells. *C*, Proliferative response of B cells stimulated with IL-4 plus: anti-CD38, anti-IgM $F(ab')_2$, LPS, or anti-CD40, in the presence (□) or absence (vehicle only, DMSO, ■) of the PKC inhibitor, Ro-318425 (62 nM). Proliferative responses were analyzed by [^{3}H]thymidine incorporation.

CD38 signaling mediates PKC-dependent activation of NF-κB

PKCs are required for the activation of the NF-κB following BCR engagement (36). We therefore next examined the effect of CD38-dependent, PKC activation on the NF-κB signaling cascade. To determine the initial step of the NF-κB pathway, IκB-α degradation was evaluated following primary B cell activation with various stimuli including anti-CD38, anti-IgM, LPS, or PMA, in the presence of cycloheximide. As expected, we observed a low basal level of IκB-α degradation in unstimulated or nonspecific rat IgG-stimulated B cells. This is consistent with the estimated 2-h $t_{1/2}$ of IκB-α in the presence of cycloheximide (37) (Fig. 4, *A* and *B*). CD38 engagement led to a marked reduction in IκB-α levels within ~60 min poststimulation with protein barely detectable by 120 min (Fig. 4*A*). Similar kinetics was also observed with both anti-IgM and LPS control stimuli. To determine whether CD38-mediated IκB-α degradation, and hence NF-κB activation, was

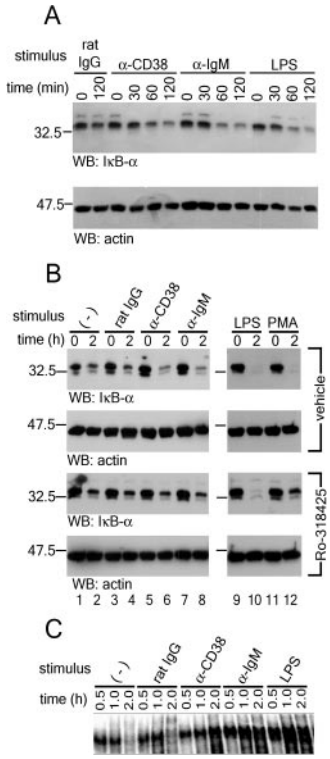

FIGURE 4. CD38 induces a PKC-dependent, IκB-α degradation and NF-κB activation in B lymphocytes. *A*, Kinetics of IκB-α degradation of cycloheximide-pretreated splenic B lymphocytes (10^7 per time) activated with rat IgG, anti-CD38, anti-IgM $F(ab')_2$, and LPS for 0, 30, 60, or 120 min. *B*, Cycloheximide-pretreated B cells were stimulated with anti-CD38, anti-IgM $F(ab')_2$, rat IgG, LPS, or PMA, in the absence (*upper panel*, vehicle) or presence (*lower panel*) of the PKC inhibitor Ro-318425. IκB-α degradation and protein-loading controls were analyzed by Western blot using anti-IκB-α and anti-actin Abs, respectively. *C*, Splenic B cells were activated as above for 0.5 or 2 h, and EMSAs were done, as described in *Materials and Methods*.

dependent on PKC activity, cells were stimulated with the indicated agonists in the absence (Fig. 4*B*, *upper panel*) or presence (Fig. 4*B*, *lower panel*) of the PKC inhibitor, Ro-318425. Under nonactivation conditions, a basal level of IκB-α degradation is observed (*upper* and *lower panels*, *lines 1–4*, compare 0 and 2 h). In contrast, CD38 or IgM stimuli each induce further IκB-α degradation at 2 h poststimulation (*upper panel*, compare *lines 2*, *4*, *6*, and *8*). The addition of the PKC inhibitor (*lower panel*) abrogated both CD38- and IgM-dependent IκB-α degradation. The level of IκB-α degradation observed under these conditions was similar to that observed under nonactivation conditions (*lower panel*, compare *lines 2*, *4*, *6*, and *8*). The specificity of Ro-318425 used in this assay was also verified by using both PKC-dependent (PMA) and independent (LPS) control stimuli (38) (Fig. 4*B*, *upper* and *lower right panels*, *lines 9–12*).

Degradation of IκB-α results in the translocation of NF-κB to the nucleus. We evaluated the nuclear translocation of NF-κB in response to CD38 stimulation using EMSAs performed with nuclear extracts from B cells stimulated with anti-CD38, anti-IgM, or LPS. CD38 engagement leads to a significant increase in nuclear NF-κB at 2 h (Fig. 4*C*). As previously reported, NF-κB translocation was clearly detected within 30 min and peaked within ~1 h following stimulation with either LPS or anti-IgM (36). Taken together, these data clearly demonstrate that CD38 is able to activate the NF-κB pathway and that this activation is PKC dependent.

CD38 induces PKC-dependent expression of cyclin-D2

Cyclin-D2 is essential for cell cycle progression in response to BCR engagement in primary splenic B cells (39, 40), and the expression of this gene product is mediated in part by NF-κB in B lymphocytes (41). We evaluated whether the CD38 signaling could regulate the inducible expression of this target protein. Splenic B lymphocytes were stimulated, and the expression of cyclin-D2 was analyzed by Western blotting (Fig. 5). As shown in Fig. 5*A*, CD38 signaling led to an increase in the expression levels of cyclin-D2 within 12 h of receptor engagement. Similar results were obtained for expression of the antiapoptotic protein, Bcl-x_L, another NF-κB-dependent target gene (data not shown). Consistent with our model, pretreatment with the PKC inhibitor, Ro-318425, abolished CD38- and IgM-induced cyclin-D2 expression (Fig. 5*B*). Conversely, up-regulation of NF-κB target genes in response to LPS stimulation was not blocked by PKC inhibition (Fig. 5*B*, and data not shown). Thus, activation of B lymphocytes by CD38 promotes NF-κB/PKC-dependent cell proliferation, and these events are most likely mediated, at least in part, by inducible expression of cyclin-D2.

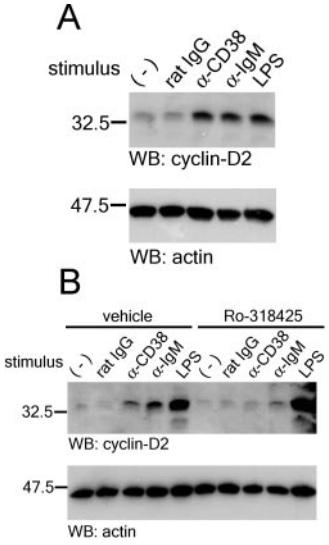

FIGURE 5. CD38 induces the PKC-dependent expression of cyclin-D2 in splenic B cells. *A*, Splenic B cells (10^7) were stimulated in the presence of 20 U/ml IL-4 with different stimuli, including LPS, anti-CD38, anti-IgM $F(ab')_2$, and rat IgG. Cyclin-D2 (12 h of stimulation) was analyzed by Western blot using rabbit-specific polyclonal Abs. *B*, Splenic B cells were stimulated as in *A* in the presence or absence of 62 nM PKC inhibitor Ro-318425; then cyclin-D2 expression was analyzed by Western blot, as above.

PC-PLC and PLD are involved in the CD38-dependent activation of splenic B lymphocytes

The requirement for DAG-dependent PKC isoforms, and the absence of PLC-γ2 activation, strongly suggested that CD38 receptor engagement promoted DAG production via an alternate, PLC-γ2-independent pathway. Recent data have suggested that several cytokine receptors as well as other cell surface receptors can mediate DAG-dependent PKC activation without activation of phosphoinositide-PLC isoforms, IP_3 production, or ER-dependent intracellular Ca^{2+} release (20–23). In these reports, it has been postulated that DAG is generated in these signaling cascades via the activation of phosphatidylcholine-PLC, PC-PLC. To directly analyze the potential for CD38-dependent activation of this enzyme in B lymphocytes, we stimulated the cells with rat IgG, anti-CD38, or anti-IgM Abs. Activated cells were lysed at different time points, and lysates were analyzed for PC-PLC activity using an indirect fluorometric measurement of phosphocholine. The increase in relative fluorescence units reflects increased activity of PC-PLC (see *Materials and Methods*) (Fig. 6*A*). Activation of B cells with anti-CD38 Ab induced a marked, time-dependent activation of PC-PLC. Activity was induced within 60 min and continued to increase at up to 180 min following receptor engagement (Fig. 6*A*). In contrast, minimal or no change in PC-PLC activation was observed following short- or long-term stimulations with anti-IgM (Fig. 6*B*, and data not shown).

To further evaluate the biological function of this enzyme in CD38 activation, we analyzed the proliferative response of B cells stimulated with either anti-CD38 or other B cell activators in the presence of D609, an inhibitor of PC-PLC. Although this inhibitor is relatively specific for PC-PLC at the concentrations used, it also exhibits inhibitory activity against other phospholipases including PLD (42) (Fig. 6*C*). Addition of D609 led to a total and dose-dependent inhibition of anti-CD38-induced proliferation. Interestingly, this inhibitor also significantly reduced anti-IgM-dependent proliferation. In contrast, LPS- and CD40-induced proliferation were not significantly affected by D609, suggesting that these stimuli do not require PC-PLC or PLD for their signaling function in primary B cells.

As anti-IgM stimulation did not lead to a significant increase in PC-PLC activation, the inhibitory effect of D609 on anti-IgM-induced proliferation is most likely the result of inhibition of other PC-specific phospholipases. Supporting this hypothesis, it has been reported that PLD is activated in lymphocytes stimulated via the BCR (43–45). Based on this evidence, we next analyzed the participation of PLD in both CD38- and IgM-dependent signaling in splenic B cells (Fig. 7, *C* and *D*; wild type (WT)). Notably, both CD38 and IgM stimulation induced the activation of PLD in splenic B cells. However, PLD-induced activation was significantly higher following CD38 stimulation in comparison with BCR engagement (Fig. 7, compare *C* and *D*; WT), suggesting a prominent role for PLD in the CD38 signaling pathway.

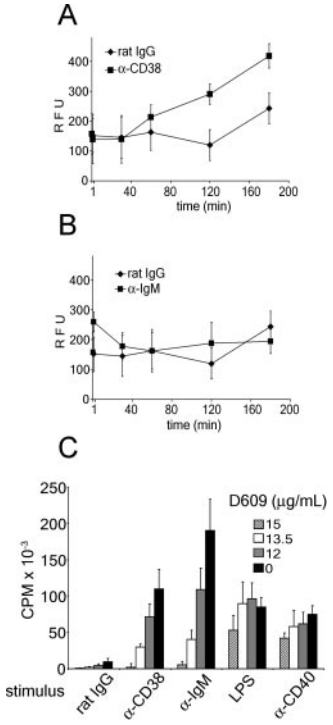

FIGURE 6. PC-PLC is activated and required for proliferation of B cells activated via CD38. Splenic B cells (10^8) were stimulated with rat IgG, anti-CD38, and anti-IgM for 0, 1, 30, 60, 120, or 180 min. Cellular lysates were prepared, and 15 μg of total proteins was analyzed for PC-PLC activity using a PC-PLC assay (for assay details, please see *Materials and Methods*). The increase in PC-PLC activity was measured as an increase in the relative fluorescence units produced by an increase in the production of resorufine. Squares represent the anti-CD38 stimulation (*A*) and anti-IgM stimulation (*B*). Diamonds represent the rat IgG as control stimulus. *C*, Splenic B cells were preincubated for 30 min with the PC-PLC inhibitor, D609, at the concentrations shown in the figure, and proliferation assays were conducted in the presence of the inhibitor with the following stimuli: rat IgG, anti-CD38, anti-IgM F(ab′)$_2$, LPS, and anti-CD40, all of them in the presence of 20 U/ml IL-4. Proliferation was measured by [^{3}H]thymidine incorporation.

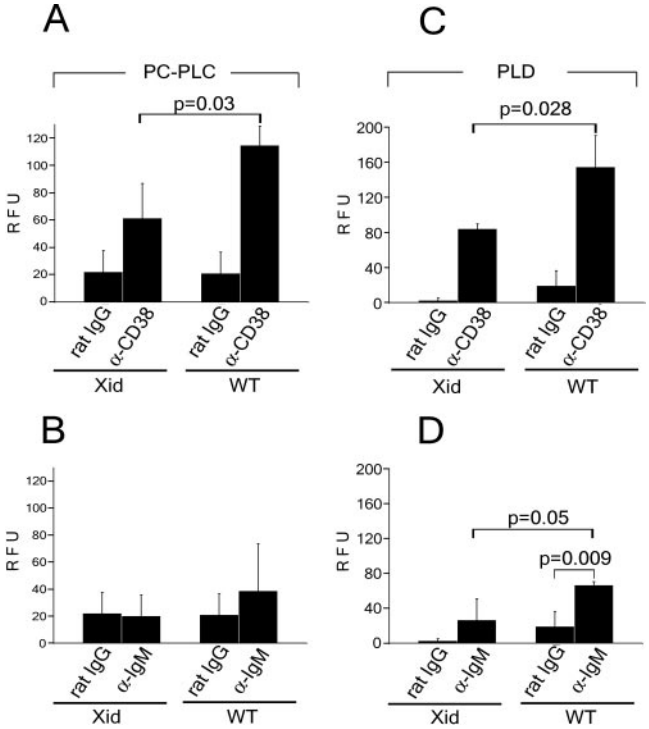

FIGURE 7. Btk is partially required for PLD and PC-PLC activation in the CD38 signaling. Splenic B cells (10^7) from BALB/c (WT) or BALB/c-XID (Xid) mice were stimulated with anti-CD38, anti-IgM Abs, or rat IgG for 180 min at 37°C. Total cell lysates were prepared, protein concentration was determined, and 15 μg of total proteins was analyzed for PC-PLC (*A* and *B*) or PLD (*C* and *D*) activity following the manufacturer instructions. Data represent mean ± SD of three independent experiments using five mice for each data point. The groups were compared with unpaired Student's *t* test using the program INSTAT 2.02 (GraphPad), in which *p* values ≤0.05 were considered to be significant. RFU, relative fluorescence units.

Based on the requirement for Btk in CD38-mediated signaling, we next evaluated whether Btk is required for the activation of PC-PLC and/or PLD following CD38 stimulation (Fig. 7). Consistent with previous reports (43, 45), we found anti-IgM-induced PLD activation was reduced in splenic B cells purified from Btk mutant, Xid mice (Fig. 7*D*). CD38-dependent activation of both PLD and PC-PLC was also reduced in Xid compared with WT B cells. Enzyme activity in Btk mutant cells was approximately one-half of the total activity in the WT cells (Fig. 7, *A* and *C*). These data clearly indicate that Btk contributes, although partially, to the CD38-dependent activation of both PLD and PC-PLC.

D609 blocks NF-κB activation and cyclin-D2 induction in the CD38-dependent signaling

We next evaluated the role for PC-PLC and PLD in downstream CD38-dependent signals. We tested the role for these enzymes in NF-κB activation, and cyclin-D2 synthesis using the PC-PLC/PLD inhibitor, D609 (Fig. 8). D609 blocked both the degradation of IκB-α (Fig. 8*A*) and the synthesis of cylin-D2 (Fig. 8*B*) in response to anti-CD38 or anti-IgM stimuli. In contrast, control, LPS-dependent cell proliferation, IκB-α degradation, and cyclin-D2 induction were not altered by this inhibitor (Fig. 8). Taken together, these findings strongly suggest that these two enzymes are crucial mediators of a CD38-dependent, Btk, PKC-dependent, and PLCγ2-independent signaling cascade leading to NF-κB activation, cyclin-D2 induction, and proliferation of mature B cells.

Discussion

In this work, we have evaluated the participation of key downstream signaling proteins in the activation of splenic B cells stimulated through the CD38 cell surface receptor. CD38 activation with agonistic Abs in B lymphocytes results in a variety of biological responses including: proliferation, survival, class switching, and apoptosis (9, 32, 46, 47). Most notably, while it is clear that the CD38-dependent signal is absolutely dependent upon Btk (12), our data demonstrate that Btk most likely mediates its crucial activity, via an alternative, PLC-γ2-independent, signaling pathway(s) that leads, directly or indirectly, to the activation of PC-PLC and PLD. These events promote the activation of PKC isoforms and NF-κB. The cascade leads to expression of cell cycle genes including cyclin-D2 and cell proliferation. Fig. 9 presents our current working model of this signaling cascade.

Recent studies of BCR-dependent signaling have demonstrated the critical role for PLC-γ2 as a primary target for Btk-dependent signals (13–15) (Fig. 9*A*). This has led to confusion with regard to the role for the PLC-γ2 pathway in CD38 signaling. For example, the absence of phosphoinositide metabolism and IP_3-dependent Ca^{2+} mobilization in CD38-stimulated splenic B cells (8, 9) seemed contradictory to the requirement for PKC activation in this pathway (32). In the current study, we clearly demonstrate that

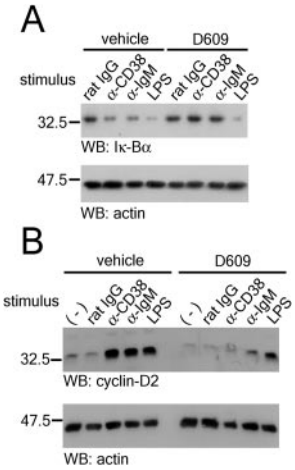

FIGURE 8. The PC-phospholipase-specific inhibitor, D609, blocks the degradation of IκB-α and the synthesis of cyclin-D2 in response to CD38 signaling. *A*, Cycloheximide-pretreated splenic B lymphocytes (10^7) from BALB/c mice were stimulated with rat IgG, anti-CD38, anti-IgM, and LPS for 120 min at 37°C, and IκB-α degradation was analyzed by Western blotting, as above. *B*, B cells (10^7 per stimulus) were activated in the presence of 20 U/ml IL-4 with different stimuli, including rat IgG, anti-CD38, anti-IgM F(ab′)$_2$, and LPS. Cells were lysed at 12 h, and cyclin-D2 expression was analyzed by Western blotting. Cells were preincubated for 30 min before stimulation without (vehicle) or with D609 (13.5 μg/ml).

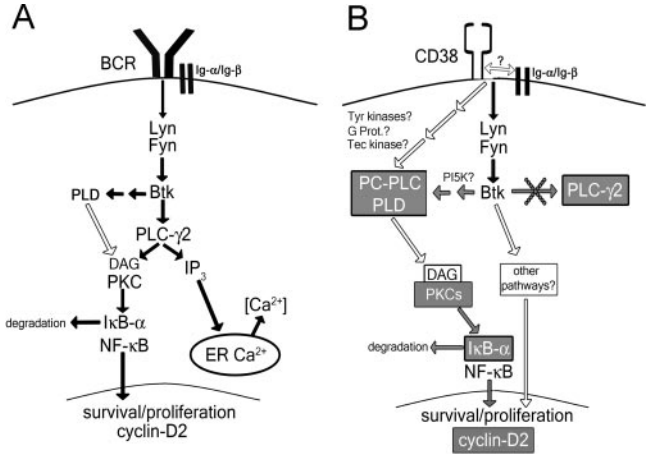

FIGURE 9. Working model of the CD38 vs BCR-dependent signaling cascade. *A*, Shows a subset of the signals triggered by BCR engagement. Activation of Src kinases, Lyn and Fyn, leads to the activation of both Syk (not shown) and Btk family kinases. Btk phosphorylates and activates PLC-γ2, generating the second messengers DAG and IP$_3$, promoting PKC activation and Ca^{2+} release, respectively. NF-κB activation is essential for cell survival, cyclin-D2 expression, and cell proliferation. In addition, BCR signaling also weakly activates PLD in a Btk-dependent manner, in which PLC-γ2 and Syk may also be involved (not shown). This additional pathway may also contribute to sustained DAG production. *B*, Shows proposed CD38 signaling pathways. CD38, probably through association with Ig-α/Ig-β subunits, activates Lyn and Fyn, leading to Btk activation. Btk does not activate PLC-γ2 and, instead, participates in association with other signals (possibly including large or small G proteins, or other tyrosine kinases) in PC-PLC/PLD activation. These events promote sustained production of DAG, and activation of PKC and NF-κB. Btk may also contribute to additional independent signals that promote cell survival, cyclin-D2 expression, and cell proliferation. Filled words and arrows designate previously reported signaling events; ▨ and gray arrows designate signaling events evaluated in this study; and □ and open arrows designate possible signals activated by CD38 cross-linking in splenic B cells. Tyr kinases, tyrosine kinases; G. prot, G proteins; PI5K, phosphatidylinositol-4-phosphate 5-kinase.

PLC-γ2 functional activity is not significantly increased following CD38 receptor engagement. This is illustrated by both the failure to induce IP$_3$ production and the lack of PLC-γ2 Y759 site-specific tyrosine phosphorylation following CD38 engagement. BCR-dependent phosphorylation of Y759 is specifically mediated by Btk, and this signal is absolutely required for full activation of PLC-γ2 (17–19). Our data suggest that unlike BCR-dependent signaling, PLC-γ2 may not be recruited into the CD38 signalosome. As BCR-dependent PLC-γ2 recruitment is largely dependent upon its interaction with the adapter protein, B cell linker (48), we anticipate that B cell linker may also be dispensable for CD38 signaling.

Although PLC-γ2 is not required for CD38 signaling, the activation of DAG-dependent PKC isoforms is clearly required for efficient signaling (Fig. 3). This was demonstrated by the reduction in the CD38-dependent proliferation observed in PKC-β-I,II knockout B cells and in Ro-318425-pretreated B cells. Although the specific PKC isoforms that participate in this pathway are not yet clear, redundant activity of several PKC isoforms appears responsible for CD38-dependent B cell proliferation. PKC-β, one of the most extensively studied isoforms in B cells (24, 49), is necessary for full CD38-dependent function. A detailed knowledge of the additional PKC isoforms involved will help to further elucidate the role of this receptor in B lymphocyte biology.

Several cytokine receptors, including the receptors for IL-1, TNF-α, IL-3, and IFN-α (20–23), are also capable of activating DAG-dependent PKC isoforms without IP$_3$ production and ER-dependent Ca^{2+} mobilization. In these receptor systems, activation of the PC-PLC appears sufficient for DAG production via metabolism of PC. Similar to these findings, our data clearly demonstrate the activation of both PC-PLC and PLD in response to CD38 receptor engagement and suggest a crucial role for these enzymatic pathways in CD38-dependent PKC activation, IκB-α degradation, cyclin-D2 synthesis, and proliferation (Figs. 6 and 8).

The activation of PLD has also been reported in response to BCR activation and to require the combined activity of Btk, Syk, and PLC-γ2 (43, 45). In contrast to these findings, our results indicate that activation of both PC-PLC and PLD can occur independently of PLC-γ2 activation, but is partially dependent on the function of Btk in response to CD38 engagement (Fig. 7). Previous reports have also shown that PLD activity is not completely abolished in Btk-deficient B cell lines stimulated with agonists including 12-*O*-tetradecanoylphorbol-13-acetate or anti-IgM (43, 50). Based upon the phenotype of Btk vs Btk/Tec-deficient B cells (51), it is likely that redundant function of an alternative Tec kinase (i.e., Tec) explains the partial requirement for Btk in PC-PLC/PLD activation. The requirement of Tec in the CD38 signaling in immature B cells (52) and its expression in mature splenic B cells (53) supports this idea. Finally, as it is reported that in addition to PLC-γ2, Btk is able to interact and/or modify additional signaling molecules including PKC, Wiskott-Aldrich syndrome protein, Cbl, Syk, and TFII-I (54), it is possible that Btk may also activate additional pathways besides PC-PLC/PLD required for the CD38-dependent B cell proliferation (Fig. 9*B*).

It remains to be determined how Tec kinases function in activation of the PC-PLC and PLD pathways. Consistent with both IgM and CD38 signaling, PKC isoforms most likely participate in the regulation of selected PLD isoforms in B cells (55). We suggest that the combined effects of a CD38-dependent transient increase in intracellular calcium levels, PC-PLC-mediated DAG production, and Btk-adapter-mediated phosphatidylinositol-4-phosphate 5-kinase membrane recruitment (56) may be sufficient

to trigger PIP_2- and classical PKC-dependent, PLD activation (55). The molecular events controlling CD38- and Btk-dependent, PC-PLC activation are even less well defined. It will be important to ascertain whether Btk functions directly or indirectly in these events and whether PC-PLC activation may also require other signaling pathways. Future studies will be required to address the specific role for signaling modules reported to be involved in both PC-PLC and PLD activation, including heterotrimeric G proteins, small G proteins, MAPKs, tyrosine kinases, and PIP_2 (55).

Our data also indicate that activation of PKC isoforms and PC-PLC/PLD by CD38 is responsible for downstream activation of the NF-κB pathway. This is supported by our observations of CD38-dependent IκB-α degradation, NF-κB nuclear translocation, and induction of the cell cycle regulator, cyclin-D2 (Figs. 4, 5, and 8). Previous reports have shown both that Btk is necessary to activate NF-κB and that NF-κB signaling is required for CD38-dependent B cell proliferation (32). This suggests that the Btk, PC-PLC/PLD, and PKC pathways together promote NF-κB activation, which through cyclin-D2 expression leads to CD38-dependent B cell proliferation (Fig. 9*B*). Interestingly, most of these pathways and specific downstream targets are similarly up-regulated in response to BCR engagement in splenic B cells and also require the activity of Btk (57). The BCR/NF-κB signaling pathway requires the activation of a CARD-MAGUK1/B cell lymphoma 10/1κB kinase signalosome specifically mediated by PKC-β-dependent CARD-MAGUK1 activation within BCR-associated lipid raft microdomains (49, 58, 59) (D. Rawlings, unpublished data). It will be important to determine whether CD38 signaling regulates NF-κB activation via a similar or distinct pathway; and whether membrane lipid rafts are also required for efficient CD38 signaling in B cells.

Notably, it remains unclear how CD38-dependent intracellular signals are initiated in splenic B cells. Previous reports indicate that deletion of the intracellular region of CD38 does not alter its signaling function (60, 61), suggesting that CD38 is likely to be physically and/or functionally associated with another cell receptor. Accordingly, it has been reported that CD38-dependent activation of T and B cell lines requires the parallel expression and function of the TCR or BCR and their corresponding signaling modules (CD3 for TCR and Ig-α/Ig-β for BCR), respectively (62–65). This observation is consistent with the requirement for Src and Tec kinases to initiate both BCR (66) and CD38 signaling in B cells. It will be important to determine whether the BCR and its signaling modules Ig-α/Ig-β are necessary to initiate the CD38-dependent signaling in primary splenic B cells. Despite the potential role for Ig-α/Ig-β in CD38 signaling, our results clearly indicate that CD38 triggers distinct signals, in which Btk, the lack of PLC-γ2 activation, and the preferential PC-PLC/PLD activation play a central role (Fig. 9).

Together, these observations suggest the opportunity to identify novel Btk-dependent substrates and/or protein interactions. Identification of PC-PLC- and PLD-dependent genes may also help to better elucidate the biological functions of these key enzymes and the PC cycle in B lymphocyte development, survival, and activation.

Acknowledgments

We thank Dr. José Manuel Hernández Hernández for technical advice, and MSc. Héctor Romero-Ramírez for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Harada, N., L. Santos-Argumedo, R. Chang, J. C. Grimaldi, F. E. Lund, C. I. Brannan, N. G. Copeland, N. A. Jenkins, A. W. Heath, R. M. Parkhouse, and M. Howard. 1993. Expression cloning of a cDNA encoding a novel murine B cell activation marker: homology to human CD38. *J. Immunol. 151:3111*.
2. Lund, F. E., D. A. Cockayne, T. D. Randall, N. Solvason, F. Schuber, and M. C. Howard. 1998. CD38: a new paradigm in lymphocyte activation and signal transduction. *Immunol. Rev. 161:79*.
3. Shubinsky, G., and M. Schlesinger. 1997. The CD38 lymphocyte differentiation marker: new insight into its ectoenzymatic activity and its role as a signal transducer. *Immunity 7:315*.
4. Berthelier, V., J. M. Tixier, H. Muller-Steffner, F. Schuber, and P. Deterre. 1998. Human CD38 is an authentic $NAD(P)^+$ glycohydrolase. *Biochem. J. 330:1383*.
5. Howard, M., J. C. Grimaldi, J. F. Bazan, F. E. Lund, L. Santos-Argumedo, R. M. Parkhouse, T. F. Walseth, and H. C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science 262:1056*.
6. Sitsapesan, R., S. J. McGarry, and A. J. Williams. 1995. Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release. *Trends Pharmacol. Sci. 16:386*.
7. Funaro, A., M. Morra, L. Calosso, M. G. Zini, C. M. Ausiello, and F. Malavasi. 1997. Role of the human CD38 molecule in B cell activation and proliferation. *Tissue Antigens 49:7*.
8. Kirkham, P. A., L. Santos-Argumedo, M. M. Harnett, and R. M. Parkhouse. 1994. Murine B-cell activation via CD38 and protein tyrosine phosphorylation. *Immunology 83:513*.
9. Santos-Argumedo, L., C. Teixeira, G. Preece, P. A. Kirkham, and R. M. Parkhouse. 1993. A B lymphocyte surface molecule mediating activation and protection from apoptosis via calcium channels. *J. Immunol. 151:3119*.
10. Yasue, T., H. Nishizumi, S. Aizawa, T. Yamamoto, K. Miyake, C. Mizoguchi, S. Uehara, Y. Kikuchi, and K. Takatsu. 1997. A critical role of Lyn and Fyn for B cell responses to CD38 ligation and interleukin 5. *Proc. Natl. Acad. Sci. USA 94:10307*.
11. Kikuchi, Y., T. Yasue, K. Miyake, M. Kimoto, and K. Takatsu. 1995. CD38 ligation induces tyrosine phosphorylation of Bruton tyrosine kinase and enhanced expression of interleukin 5-receptor α chain: synergistic effects with interleukin 5. *Proc. Natl. Acad. Sci. USA 92:11814*.
12. Santos-Argumedo, L., F. E. Lund, A. W. Heath, N. Solvason, W. W. Wu, J. C. Grimaldi, R. M. Parkhouse, and M. Howard. 1995. CD38 unresponsiveness of xid B cells implicates Bruton's tyrosine kinase (*btk*) as a regular of CD38 induced signal transduction. *Int. Immunol. 7:163*.
13. Fluckiger, A. C., Z. Li, R. M. Kato, M. I. Wahl, H. D. Ochs, R. Longnecker, J. P. Kinet, O. N. Witte, A. M. Scharenberg, and D. J. Rawlings. 1998. Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. *EMBO J. 17:1973*.
14. Petro, J. B., and W. N. Khan. 2001. Phospholipase C-γ2 couples Bruton's tyrosine kinase to the NF-κB signaling pathway in B lymphocytes. *J. Biol. Chem. 276:1715*.
15. Takata, M., and T. Kurosaki. 1996. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. *J. Exp. Med. 184:31*.
16. Hempel, W. M., R. C. Schatzman, and A. L. DeFranco. 1992. Tyrosine phosphorylation of phospholipase C-γ2 upon cross-linking of membrane Ig on murine B lymphocytes. *J. Immunol. 148:3021*.
17. Rodriguez, R., M. Matsuda, O. Perisic, J. Bravo, A. Paul, N. P. Jones, Y. Light, K. Swann, R. L. Williams, and M. Katan. 2001. Tyrosine residues in phospholipase Cγ 2 essential for the enzyme function in B-cell signaling. *J. Biol. Chem. 276:47982*.
18. Watanabe, D., S. Hashimoto, M. Ishiai, M. Matsushita, Y. Baba, T. Kishimoto, T. Kurosaki, and S. Tsukada. 2001. Four tyrosine residues in phospholipase C-γ2, identified as Btk-dependent phosphorylation sites, are required for B cell antigen receptor-coupled calcium signaling. *J. Biol. Chem. 276:38595*.
19. Humphries, L. A., C. Dangelmaier, K. Sommer, K. Kipp, R. M. Kato, N. Griffith, I. Bakman, C. W. Turk, J. L. Daniel, and D. J. Rawlings. 2004. Tec kinases mediate sustained calcium influx via site-specific tyrosine phosphorylation of the phospholipase Cγ Src homology 2-Src homology 3 linker. *J. Biol. Chem. 279:37651*.
20. Pfeffer, L. M., B. Strulovici, and A. R. Saltiel. 1990. Interferon-α selectively activates the β isoform of protein kinase C through phosphatidylcholine hydrolysis. *Proc. Natl. Acad. Sci. USA 87:6537*.
21. Rao, P., and R. A. Mufson. 1994. Human interleukin-3 stimulates a phosphatidylcholine specific phospholipase C and protein kinase C translocation. *Cancer Res. 54:777*.
22. Rosoff, P. M., N. Savage, and C. A. Dinarello. 1988. Interleukin-1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell 54:73*.
23. Schutze, S., D. Berkovic, O. Tomsing, C. Unger, and M. Kronke. 1991. Tumor necrosis factor induces rapid production of 1′2′diacylglycerol by a phosphatidylcholine-specific phospholipase C. *J. Exp. Med. 174:975*.
24. Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky. 1996. Immunodeficiency in protein kinase Cβ-deficient mice. *Science 273:788*.
25. Heath, A. W., W. W. Wu, and M. C. Howard. 1994. Monoclonal antibodies to murine CD40 define two distinct functional epitopes. *Eur. J. Immunol. 24:1828*.
26. Dyer, R. B., and N. K. Herzog. 1995. Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. *BioTechniques 19:192*.
27. Reyes-Reyes, M., N. Mora, A. Zentella, and C. Rosales. 2001. Phosphatidylinositol 3-kinase mediates integrin-dependent NF-κB and MAPK activation through separate signaling pathways. *J. Cell Sci. 114:1579*.

28. Wang, D., J. Feng, R. Wen, J. C. Marine, M. Y. Sangster, E. Parganas, A. Hoffmeyer, C. W. Jackson, J. L. Cleveland, P. J. Murray, and J. N. Ihle. 2000. Phospholipase Cγ2 is essential in the functions of B cell and several Fc receptors. *Immunity 13:25*.
29. Jou, S. T., N. Carpino, Y. Takahashi, R. Piekorz, J. R. Chao, N. Carpino, D. Wang, and J. N. Ihle. 2002. Essential, nonredundant role for the phosphoinositide 3-kinase p110δ in signaling by the B-cell receptor complex. *Mol. Cell. Biol. 22:8580*.
30. Hashimoto, A., K. Takeda, M. Inaba, M. Sekimata, T. Kaisho, S. Ikehara, Y. Homma, S. Akira, and T. Kurosaki. 2000. Cutting edge: essential role of phospholipase C-γ2 in B cell development and function. *J. Immunol. 165:1738*.
31. Zubiaur, M., M. Izquierdo, C. Terhorst, F. Malavasi, and J. Sancho. 1997. CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-ζ/ζ-associated protein-70 signaling pathways in Jurkat T lymphocytes. *J. Immunol. 159:193*.
32. Kaku, H., K. Horikawa, Y. Obata, I. Kato, H. Okamoto, N. Sakaguchi, S. Gerondakis, and K. Takatsu. 2002. NF-κB is required for CD38-mediated induction of Cγ1 germline transcripts in murine B lymphocytes. *Int. Immunol. 14:1055*.
33. Newton, A. C. 1995. Protein kinase C: structure, function, and regulation. *J. Biol. Chem. 270:28495*.
34. Lalmanach-Girard, A. C., T. C. Chiles, D. C. Parker, and T. L. Rothstein. 1993. T cell-dependent induction of NF-κB in B cells. *J. Exp. Med. 177:1215*.
35. Lapointe, R., R. Lemieux, M. Olivier, and A. Darveau. 1996. Tyrosine kinase and cAMP-dependent protein kinase activities in CD40-activated human B lymphocytes. *Eur. J. Immunol. 26:2376*.
36. Liu, J. L., T. C. Chiles, R. J. Sen, and T. L. Rothstein. 1991. Inducible nuclear expression of NF-κB in primary B cells stimulated through the surface Ig receptor. *J. Immunol. 146:1685*.
37. Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben Neriah, and P. A. Baeuerle. 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature 365:182*.
38. Bone, H., and N. A. Williams. 2001. Antigen-receptor cross-linking and lipopolysaccharide trigger distinct phosphoinositide 3-kinase-dependent pathways to NF-κB activation in primary B cells. *Int. Immunol. 13:807*.
39. Solvason, N., W. W. Wu, D. Parry, D. Mahony, E. W. Lam, J. Glassford, G. G. Klaus, P. Sicinski, R. Weinberg, Y. J. Liu, et al. 2000. Cyclin D2 is essential for BCR-mediated proliferation and CD5 B cell development. *Int. Immunol. 12:631*.
40. Chiles, T. C. 2004. Regulation and function of cyclin D2 in B lymphocyte subsets. *J. Immunol. 173:2901*.
41. Hinz, M., P. Loser, S. Mathas, D. Krappmann, B. Dorken, and C. Scheidereit. 2001. Constitutive NF-κB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. *Blood 97:2798*.
42. Kiss, Z., and M. Tomono. 1995. Compound D609 inhibits phorbol ester-stimulated phospholipase D activity and phospholipase C-mediated phosphatidylethanolamine hydrolysis. *Biochim. Biophys. Acta 1259:105*.
43. Forssell, J., A. Nilsson, and P. Sideras. 2000. Reduced formation of phosphatidic acid upon B-cell receptor triggering of mouse B-lymphocytes lacking Bruton's tyrosine kinase. *Scand. J. Immunol. 52:30*.
44. Gilbert, J. J., T. R. Pettitt, S. D. Seatter, S. D. Reid, M. J. Wakelam, and M. M. Harnett. 1998. Antagonistic roles for phospholipase D activities in B cell signaling: while the antigen receptors transduce mitogenic signals via a novel phospholipase D activity, phosphatidylcholine-phospholipase D mediates antiproliferative signals. *J. Immunol. 161:6575*.
45. Hitomi, T., S. Yanagi, R. Inatome, and H. Yamamura. 1999. Cross-linking of the B cell receptor induces activation of phospholipase D through Syk, Btk and phospholipase C-γ2. *FEBS Lett. 445:371*.
46. Horikawa, K., H. Kaku, H. Nakajima, H. W. Davey, L. Henninghausen, I. Iwamoto, T. Yasue, A. Kariyone, and K. Takatsu. 2001. Essential role of Stat5 for IL-5-dependent IgH switch recombination in mouse B cells. *J. Immunol. 167:5018*.
47. Kitanaka, A., C. Ito, H. Nishigaki, and D. Campana. 1996. CD38-mediated growth suppression of B-cell progenitors requires activation of phosphatidylinositol 3-kinase and involves its association with the protein product of the c-*cbl* proto-oncogene. *Blood 88:590*.
48. Ishiai, M., H. Sugawara, M. Kurosaki, and T. Kurosaki. 1999. Cutting edge: association of phospholipase C-γ2 Src homology 2 domains with BLNK is critical for B cell antigen receptor signaling. *J. Immunol. 163:1746*.
49. Su, T. T., B. Guo, Y. Kawakami, K. Sommer, K. Chae, L. A. Humphries, R. M. Kato, S. Kang, L. Patrone, R. Wall, et al. 2002. PKC-β controls I κB kinase lipid raft recruitment and activation in response to BCR signaling. *Nat. Immunol. 3:780*.
50. Hitomi, T., S. Yanagi, R. Inatome, J. Ding, T. Takano, and H. Yamamura. 2001. Requirement of Syk-phospholipase C-γ2 pathway for phorbol ester-induced phospholipase D activation in DT40 cells. *Genes Cells 6:475*.
51. Ellmeier, W., S. Jung, M. J. Sunshine, F. Hatam, Y. Xu, D. Baltimore, H. Mano, and D. R. Littman. 2000. Severe B cell deficiency in mice lacking the *tec* kinase family members Tec and Btk. *J. Exp. Med. 192:1611*.
52. Kitanaka, A., H. Mano, M. E. Conley, and D. Campana. 1998. Expression and activation of the nonreceptor tyrosine kinase Tec in human B cells. *Blood 91:940*.
53. Tomlinson, M. G., L. P. Kane, J. Su, T. A. Kadlecek, M. N. Mollenauer, and A. Weiss. 2004. Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec. *Mol. Cell. Biol. 24:2455*.
54. Qiu, Y., and H. J. Kung. 2000. Signaling network of the Btk family kinases. *Oncogene 19:5651*.
55. Exton, J. H. 1994. Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta 1212:26*.
56. Saito, K., K. F. Tolias, A. Saci, H. B. Koon, L. A. Humphries, A. Scharenberg, D. J. Rawlings, J. P. Kinet, and C. L. Carpenter. 2003. BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity 19:669*.
57. Su, T. T., and D. J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J. Immunol. 168:2101*.
58. Jun, J. E., L. E. Wilson, C. G. Vinuesa, S. Lesage, M. Blery, L. A. Miosge, M. C. Cook, E. M. Kucharska, H. Hara, J. M. Penninger, et al. 2003. Identifying the MAGUK protein Carma-1 as a central regulator of humoral immune responses and atopy by genome-wide mouse mutagenesis. *Immunity 18:751*.
59. Newton, K., and V. M. Dixit. 2003. Mice lacking the CARD of CARMA1 exhibit defective B lymphocyte development and impaired proliferation of their B and T lymphocytes. *Curr. Biol. 13:1247*.
60. Kitanaka, A., T. Suzuki, C. Ito, H. Nishigaki, E. Coustan-Smith, T. Tanaka, Y. Kubota, and D. Campana. 1999. CD38-mediated signaling events in murine pro-B cells expressing human CD38 with or without its cytoplasmic domain. *J. Immunol. 162:1952*.
61. Lund, F. E., H. M. Muller-Steffner, N. Yu, C. D. Stout, F. Schuber, and M. C. Howard. 1999. CD38 signaling in B lymphocytes is controlled by its ectodomain but occurs independently of enzymatically generated ADP-ribose or cyclic ADP-ribose. *J. Immunol. 162:2693*.
62. Lund, F. E., N. W. Solvason, M. P. Cooke, A. W. Health, J. C. Grimaldi, R. M. Parkhouse, C. C. Goodnow, and M. C. Howard. 1995. Signaling through murine CD38 is impaired in antigen receptor-unresponsive B cells. *Eur. J. Immunol. 25:1338*.
63. Lund, F. E., N. Yu, K. M. Kim, M. Reth, and M. C. Howard. 1996. Signaling through CD38 augments B cell antigen receptor (BCR) responses and is dependent on BCR expression. *J. Immunol. 157:1455*.
64. Morra, M., M. Zubiaur, C. Terhorst, J. Sancho, and F. Malavasi. 1998. CD38 is functionally dependent on the TCR/CD3 complex in human T cells. *FASEB J. 12:581*.
65. Zubiaur, M., M. Guirado, C. Terhorst, F. Malavasi, and J. Sancho. 1999. The CD3-γδε transducing module mediates CD38-induced protein-tyrosine kinase and mitogen-activated protein kinase activation in Jurkat T cells. *J. Biol. Chem. 274:20633*.
66. Gauld, S. B., J. M. Dal Porto, and J. C. Cambier. 2002. B cell antigen receptor signaling: roles in cell development and disease. *Science 296:1641*.