Phospholipase C-γ2 Couples Bruton’s Tyrosine Kinase to the NF-κB Signaling Pathway in B Lymphocytes

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The generation and survival of B lymphocyte subpopulations is contingent upon the expression of a functional B cell antigen receptor complex (BCR)1 (1, 2). BCR engagement directs B cell biological responses by initiating biochemical signaling cascades involving the cytoplasmic protein tyrosine kinases Lyn, Syk, and BTK (3–5). BTK plays an integral role in transducing BCR-directed signals, because mutations in the btk gene result in the B cell deficiencies X-linked agammaglobulinemia (XLA) in man and X-linked immunodeficiency (xid) in mice (6–10). B cells from xid mice are defective in survival and proliferation, implicating BTK in these biological processes (10–12). However, the molecular mechanisms by which BTK effects B cell proliferation and survival are not well understood. Like BTK, transcription factor NF-κB has been implicated in the regulation of genes essential for B cell responses including proliferation and survival (13–15). In resting cells, NF-κB is sequestered in the cytoplasmic compartment via its association with a family of inhibitory proteins, termed IκBs (16). Recent studies have identified a cytokine-inducible IκB kinase complex (IKK) consisting of two catalytic IκKα and IκKβ and one regulatory subunit (IKKγ) (17). In response to NF-κB activating signals, IκK phosphorylates and targets IκB for degradation (17). We and others (18, 19) have recently shown that BTK couples the BCR to IκK and NF-κB. However, the biochemical mechanism by which BTK activates NF-κB remains largely undefined.

BTK, in concert with the protein tyrosine kinase Syk and the adaptor protein BLNK, has recently been demonstrated to phosphorylate and activate PLC-γ2 (22–24). In response to BCR signals, PLC-γ2 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate induces the release of Ca2+ from intracellular stores, and diacylglycerol facilitates the activation of PKC isoenzymes (20, 21). Thus, BTK-dependent activation of PLC-γ2 is essential for BCR-initiated calcium fluxes (22). However, the functional consequences of PLC-γ2 signaling in the activation of nuclear factors that direct B cell responses are not known.

In this report, we provide two lines of evidence indicating that BCR-initiated activation of NF-κB is mediated by PLC-γ2. First, DT40 chicken B cells deficient for PLC-γ2 fail to translocate NF-κB to the nucleus upon BCR activation. Second, pharmacologic inhibition of PLC-γ2 or its second messengers prevents BCR-responsive activation of IκK and phosphorylation of IκBo in primary B cells. These biochemical findings provide a potential molecular explanation for the B cell defects recently reported for plc-γ2−/− mice, which display an xid-like phenotype reminiscent of animals lacking functional BTK (10).

MATERIALS AND METHODS

Cells and Reagents—The chicken B cell line DT40, DT40 cells deficient for either BTK or PLC-γ2 (DT40 BTK−, DT40 PLC-γ2−) or mutant DT40 cells reconstituted with either human BTK or PLC-γ2 (DT40.BTKR, DT40.PLC-γ2R) were a kind gift of Dr. Tomohiro Kurosaki, Riken Cell Bank, Japan (23, 24). DT40 cells were maintained as described previously and were cultured in low serum media (RPMI with 0.5% FCS, 0.05% chicken serum) for 8–12 h prior to stimulation (18). Splenocytes and primary B lymphocytes were isolated from spleens of C57Bl6 mice. For phopho-IκBo Western analyses, RBC-depleted splenocytes were cultured and stimulated as indicated. For IKK in vitro kinase assays, B cells were purified by a process of negative selection on the B axis following BCR ligation. Interference with this NF-κB cascade may account for some of the B cell defects reported for plc-γ2−/− mice, which develop an X-linked immunodeficiency-like phenotype.

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‡ The abbreviations used are: BCR, B cell antigen receptor; xid, X-linked agammaglobulinemia; BTK, Bruton’s tyrosine kinase; PLC-γ2, phospholipase C-γ2; NF-κB, nuclear factor-κB; IκK, IκB kinase; PKC, protein kinase C; FMA, phorbol 12-myristate 13-acetate; Bis I, bisindolylmaleimide I; CsA, cyclosporin A; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester; GST, glutathione S-transferase; EMSA, electrophotorective mobility shift assays; PAGE, polyacrylamide gel electrophoresis; MAPK, mitogen-activated protein kinase.

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been described previously (25).

Western Blot Analyses—For Western blot analysis of RelA and c-Rel, nuclear extracts equivalent to 2 × 10^6 cells were denatured in Laemmli reducing buffer by boiling at 95 °C for 3 min, and the proteins were resolved by SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes and subjected to immunoblotting with rabbit polyclonal antibodies against RelA, c-Rel, or SP1 as described previously (18). For IkB degradation assays, 4 × 10^6 cells/sample were preincubated for 30 min in medium containing 50 µM cycloheximide and then stimulated as indicated. Cell extracts were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, probed with antibodies against chicken IkBα (pp40; gift of C. Chen) and p38 MAPK (Santa Cruz Biotechnology), and detected using the ECL system. Western blot analyses of IkBα phosphorylation were performed as above and probed with antibodies against mouse IkBα (Santa Cruz Biotechnology) or phosphorylated Ser-32/Ser-36 IkBα (Santa Cruz Biotechnology).

The indicated DT40 cell lines were each cotransfected by electroporation (26). To define further the mechanism employed by BTK to activate NF-κB, cells that were not stimulated were also incubated in medium containing 10% serum. To monitor any effects of serum on the activation of NF-κB, cells were preincubated with 50 µM cycloheximide and then were left unstimulated or stimulated as indicated. Nuclear extracts were used in EMSA. Autoradiograms were quantitated with Image Gauge software (Koshin graphics system), and levels of nuclear NF-κB were normalized against NF-Y. Quantitative results for each sample are reported as the percentage of NF-κB nucleoprotein complexes present relative to anti-IgM-treated B cells (lane 2).

In Vitro Kinase Assays—In vitro kinase assays were performed on the cytosolic fraction of 5 × 10^6 B cells as described previously (18). Briefly, cell extracts from 0.5 × 10^6 cell equivalents were removed for Western blot analysis, and the remaining cell extract was subjected to immunoprecipitation with anti-IKKα plus anti-IKKβ antibodies (Santa Cruz Biotechnology). The immunocomplexes were then resuspended in 20 µl of kinase buffer (20 mM HEPES, pH 7.2, MgCl2 (2 mM), MnCl2 (2 mM), dithiothreitol (1 mM), ATP (20 µM)) containing 1.0 µCi of [γ-^32P]ATP and 50 µg/ml wild type GST-IκBα substrate. The reaction was allowed to continue for 30 min at 30 °C under agitation and then was terminated by the addition of 4 × SDS sample buffer. The samples were resolved by 8% SDS-PAGE and stained with Coomassie Brilliant Blue to visualize the GST-IκBα substrate. The gels were dried and exposed to x-ray film to visualize γ-^32P-phosphorylated GST-IκBα.

RESULTS AND DISCUSSION

In prior studies, we established that BTK is required for nuclear translocation of NF-κB in BCR-stimulated B cells (18). However, the molecular mechanism by which BTK facilitates NF-κB activation is poorly defined. Recent findings suggest that BCR-directed nuclear translocation of NF-κB requires the activation of the calcium-responsive phosphatase calcineurin (26). To define further the mechanism employed by BTK to effect NF-κB activation, we investigated a role for calcium and calcineurin in BCR-responsive nuclear translocation of NF-κB in DT40 B cells.

The DT40 B cell system is amenable to genetic manipulation and has thus proven invaluable for biochemical analysis of BCR-signaling events (27). To determine whether calcium and calcineurin play a role in BCR-responsive activation of NF-κB in this cellular background, EMSA analyses were performed on nuclear extracts prepared from DT40 cells preincubated with pharmacological inhibitors of calcium, calcineurin, and PKCs. We used BAPTA-AM/EGTA to chelate intra- and extracellular calcium and cyclosporin A (CsA) to inhibit the calcium-responsive phosphatase calcineurin or bisindolylmaleimide (Bis 1), a broad spectrum inhibitor of PKC isoenzymes (Fig. 1). We also treated DT40 cells with PMA and ionomycin, pharmacological agents known to activate NF-κB via IKK, as a positive control (28). As expected, BCR cross-linking or PMA/ionomycin treatment resulted in the rapid nuclear accumulation of NF-κB (compare lane 1 with 2 and 10). However, BCR-directed nuclear translocation of NF-κB was inhibited by treatment with BAPTA-AM/EGTA or CsA (lanes 3 and 4). Bis 1 treatment significantly, although not completely, inhibited this response (lane 5). However, preincubation with Bis in combination with either BAPTA-AM/EGTA or CsA resulted in a complete block in NF-κB nuclear translocation upon BCR activation (lanes 6 and 7). This result demonstrates that inhibition of either calcium or calcineurin and PKC completely abolishes BCR-directed activation of NF-κB in DT40 B cells.

Upon BCR ligation, BTK activates a distinct set of signal transducers to initiate downstream signaling events (3, 29). Of these, Akt, MAPK, and PLC-γ2 have the capability to activate NF-κB via IKK. Although both Akt and MAPK have been directly linked to IKK activation (30, 31), such a role has not been demonstrated for PLC-γ2. However, our finding that calcium and PKC are essential for nuclear translocation of NF-κB in BCR-stimulated DT40 cells implicates PLC-γ2 in this response (Fig. 1). Therefore, we next explored an involvement of PLC-γ2 in NF-κB nuclear translocation in B cells stimulated via the BCR.

To determine whether PLC-γ2 is critical for BCR-directed nuclear translocation of NF-κB, we used mutant chicken DT40 B cells lacking PLC-γ2 (DT40.PLC-γ2) along with BTK-deficient (DT40.BTK) and parental DT40 B cells. Cells were induced via the BCR, and their nuclear NF-κB content was assessed by EMSA (Fig. 2A). Although BCR cross-linking leads to a marked increase in nuclear NF-κB in DT40 cells (Fig. 2A,
compare lanes 1 and 4), both DT40.PLC-\(\gamma\)2 and DT40.BTK B cells failed to demonstrate this response (Fig. 2A, compare lanes 2 and 5 and 3 and 6). However, PMA and ionomycin mobilized similar levels of nuclear NF-\(\kappa\)B in all three cell types (Fig. 2A, lanes 7–9). These results strongly suggest that like BTK, PLC-\(\gamma\)2 plays an essential role in the transmission of BCR signals to activate NF-\(\kappa\)B.

To ascertain whether the observed defect was due to delayed kinetics of NF-\(\kappa\)B activation, we compared BCR-responsive nuclear translocation of NF-\(\kappa\)B in DT40.PLC-\(\gamma\)2 cells with that in DT40 B cells over a period of 4 h (Fig. 2B). Upon BCR cross-linking, DT40 B cells rapidly translocated NF-\(\kappa\)B to the nucleus and maintained elevated levels up to 4 h after activation. In contrast, nuclear levels of NF-\(\kappa\)B did not increase in DT40.PLC-\(\gamma\)2 B cells at any time point within that period (Fig. 2B, compare lanes, 1, 3, 5, 7, and 9 with 2, 4, 6, and 8). To verify further that the NF-\(\kappa\)B activation defect in DT40.PLC-\(\gamma\)2 B cells was due to PLC-\(\gamma\)2 deficiency, reconstitution experiments were performed. In response to BCR engagement, DT40.PLC-\(\gamma\)2 B cells expressing wild type human PLC-\(\gamma\)2 (DT40.PLC-\(\gamma\)2R (23)) were capable of NF-\(\kappa\)B nuclear translocation as determined by EMSA and a NF-\(\kappa\)B responsive luciferase reporter assay (Fig. 2, C and D). These data strongly suggest that PLC-\(\gamma\)2 is critical for transmission of BCR-dependent signals that lead to the nuclear translocation of NF-\(\kappa\)B.

Members of the NF-\(\kappa\)B/Rel family of proteins include p50/NF-\(\kappa\)B1, p52/NF-\(\kappa\)B2, RelA, c-Rel, and RelB, which have the capacity to form either homo- or heterodimers (16).
dimers containing the Rel family proteins RelA or c-Rel have been demonstrated to be the principal transactivating species activated in response to BCR engagement in B cells (35). We previously demonstrated that RelA and c-Rel fail to undergo nuclear translocation upon BCR stimulation in BTK-deficient B cells. To test whether BTK-mediated RelA and c-Rel nuclear translocation requires PLC-γ2, we compared the ability of DT40.PLC-γ2, DT40.BTK, and DT40 B cells to translocate these subunits to the nucleus upon BCR-cross-linking (Fig. 3, A and B). Immunoblotting of nuclear extracts from unactivated (lanes 1–3), anti-IgM stimulated (lanes 4–6), and PMA/ionomycin treated (lanes 7–9) cells with Rel subunit-specific antibodies revealed that nuclear accumulation of both RelA and c-Rel occurs in DT40 B cells following BCR stimulation (Fig. 3, A and B, lanes 1 and 4). In contrast, BCR-responsive nuclear translocation of RelA and c-Rel is not observed in either DT40.PLC-γ2 or DT40.BTK B cells. Treatment with PMA/ionomycin induced nuclear translocation of both Rel species (Fig. 3, A and B, lanes 7–9) in all three cell lines. Furthermore, the observed differences in Rel subunit translocation are not attributable to either variation in total protein content of the nuclear extracts or their integrity, because similar amounts of p38 MAPK to verify the protein content and integrity.

NF-κB dimers are found in the cytoplasm of quiescent cells, bound to members of a family of inhibitory molecules termed IκBα. BCR-induced nuclear translocation of NF-κB is contingent upon the phosphorylation and proteolytic degradation of IκBα, a process that requires BTK. We compared the ability of DT40.PLC-γ2 B cells with DT40.BTK and DT40 B cells to degrade IκBα in response to BCR activation. Cells were incubated with anti-IgM antibodies or PMA and ionomycin for indicated periods, and cytoplasmic extracts were immunoblotted for chicken IκBα (Fig. 4, upper panel). As expected, DT40 B cells rapidly degraded IκBα upon BCR activation. Consistent with the results shown in Fig. 2, DT40.PLC-γ2 B cells failed to degrade IκBα in response to BCR stimulation (Fig. 4, compare lanes 1–4 with 6–9 and 11–14). All three cell lines efficiently degraded IκBα in response to treatment with PMA and ionomycin. Therefore, loss of PLC-γ2 does not affect the downstream components necessary for IκBα degradation. These results demonstrate that BCR-directed degradation of IκBα specifically requires PLC-γ2.

Prior biochemical studies have identified several NF-κB agonists that converge on IKKα and IKKβ including TNF and IL-1β (17). Additionally, we have recently established that BCR-initiated activation of NF-κB by BTK proceeds via IKKα (18). To extend our finding that PLC-γ2 is required for BCR-directed nuclear translocation of NF-κB, we explored a role for PLC-γ2 in IKK activation. We tested whether pharmacological agents that block PLC-γ2 and its second messengers could prevent BCR-induced activation of IKK in primary B cells (Fig. 5A). In response to activation signals via the BCR or CD40, or treatment with PMA, IKK enzymatic activity was significantly increased as determined by in vitro kinase assays using recombinant GST-IκBα as the substrate (Fig. 5A, compare lane 1 with 2, 7, and 8). In contrast, incubation of B cells with either the PLC-γ-specific inhibitor (U-73122) or inhibitors of its second messengers (BAPTA-AM/EGTA, CsA, or Bis I) prior to BCR stimulation abolished this activity (Fig. 5A, lanes 3–6). These data implicate PLC-γ2, calcium, calcineurin, and PKC in IKK activation upon BCR ligation. Moreover, they verify the role of these signaling molecules in BCR-responsive activation of IKK in a physiologically relevant background.

To confirm this observation, we performed Western blot analyses of cytosolic fractions from BCR-, CD40-, or PMA-stimulated splenocytes using an antibody directed against Ser32/Ser36-phosphorylated IκBα (Fig. 5B, upper panel). Stimulation via either the BCR or CD40-induced phosphorylation of IκBα (Fig. 5B, compare lanes 1, 2, and 6). BCR-induced IκBα phosphorylation was blocked by pretreatment with either BAPTA-AM/EGTA, CsA, or Bis I (Fig. 5B, lanes 3–5). Also, PMA stimulation resulted in IκBα phosphorylation that was abrogated by pretreatment with the PKC inhibitor Bis I (Fig. 5B, lanes 7 and 8). These observations implicate PLC-γ2, calcium, and PKC in BCR-responsive activation of IKK and phosphorylation of IκBα. Moreover, the observation that cells pre-
treated with CsA fail to activate IKK upon BCR cross-linking identifies calcineurin as a critical mediator of this response. This observation is consistent with the recent finding that calcineurin and PKCs synergize to induce IKK activation in T cells (28). Collectively, these data suggest that PLC-γ2 is likely to mediate BCR-responsive activation of IKK, phosphorylation of IxBα, and nuclear translocation of NF-κB.

We have found that PLC-γ2 and its downstream signals are essential for BCR-directed activation of IKK and NF-κB. Prior studies in BCR-stimulated B cells have revealed that PLC-γ2 is activated via the concerted actions of BTK, Syk, and BLNK (3, 32). Therefore, it is likely that PLC-γ2 is the principal BTK signal transducer for BCR-directed activation of IKK and NF-κB. Further investigation is required to determine whether additional BTK targets, including Akt and MAPK, synergize with PLC-γ2 to effect nuclear translocation of NF-κB in BCR-stimulated B cells. However, the placement of PLC-γ2 in the BCR/BTK/NF-κB signaling pathway provides the first potential molecular explanation for the similar xid-like B cell deficiencies displayed by plc-γ2−/− and btk−/− mice (10, 33).

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REFERENCES

1. Lam, K. P., Kuhn, R., and Rajewsky, K. (1997) Cell 90, 1073–1083
2. Rajewsky, K. (1996) Nature 381, 753–758
3. Fruman, D. A., Satterthwaite, A. B., and Witte, O. N. (2000) Immunity 13, 1–3
4. Yang, W. C., Collette, Y., Nunes, J. A., and Olive, D. (2000) Immunity 12, 373–382
5. Campbell, K. S. (1999) Curr. Opin. Immunol. 11, 256–264
6. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarström, L., Kinmon, C., Levinsky, R., Bobrow, M., Smith, C. I., and Bentley, D. R. (1993) Nature 361, 226–233
7. Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., Sparkes, R. S., Kubagawa, H., Moshandas, T., Quan, S., Belmont, B. W., Cooper, M. D., Conley, M. E., and Witte, O. N. (1993) Cell 72, 279–290
8. Thomas, J. D., Sideras, P., Smith, C. I., Vorechovsky, I., Chapman, V., and Paul, W. E. (1993) Science 261, 355–358
9. Rawlings, D. J., Saffran, D. C., Tsukada, S., Large, J. E., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., Jenkins, N. A., and Witte, O. N. (1993) Science 261, 358–361
10. Khan, W. N., Ati, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kent, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998) EMBO J. 17, 1973–1985
11. Anderson, J. S., Teutch, M., Dong, Z., and Wortis, H. H. (1996) J. Exp. Med. 187, 1081–1091
12. Solvsønn, N., Wu, W., Kabra, N., Lund-Johansen, P., Roncarolo, M. G., Behrenz, T. W., Grillot, D. A., Nunes, G., Lees, E., and Howard, M. (1998) J. Exp. Med. 187, 663–674
13. Bendall, H. H., Sikes, M. L., Ballard, D. W., and Oltz, E. M. (1999) Mol. Immunol. 36, 187–195
14. Grumont, R. J., Rourke, I. J., Shi, W., Miyake, K., Ohs, H. D., Longnecker, R., Kinet, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998) EMBO J. 17, 1973–1985
15. Kontgen, F., Grumont, R. J., Strasser, A., Metcalf, D. L., Ri, L., Tarlinton, D., and Gerondakis, S. (1995) Genes Dev. 9, 1963–1977
16. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
17. Zandi, E., and Karin, M. (1999) Mol. Cell. Biol. 19, 4547–4551
18. Petro, J. B., Rahman, S. M., Ballard, D. W., and Khan, W. N. (2000) J. Exp. Med. 191, 1745–1754
19. Bajpai, U. D., Zhang, K., Teutsch, M., Sen, R., and Wortis, H. H. (2000) J. Exp. Med. 191, 1735–1744
20. Berridge, M. J. (1993) Nature 361, 315–325
21. Imboden, J. B., and Stobo, J. D. (1985) J. Exp. Med. 161, 446–456
22. Flickinger, A. C., Li, Z., Kato, R. M., Wahl, M. I., Oehs, H. D., Longnecker, R., Kinet, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998) J. Biol. Chem. 273, 22923–22931
23. Takata, M., Homma, Y., and Kurosaki, T. (1995) J. Exp. Med. 182, 907–914
24. Takata, M., and Kurosaki, T. (1996) J. Exp. Med. 184, 31–40
25. Chen, C. L., Yull, F. E., and Kerr, L. D. (1999) Biochem. Biophys. Res. Commun. 257, 798–806
26. Venkataraman, L., Burakoff, S. J., and Sen, R. (1995) J. Exp. Med. 181, 1091–1099
27. Kurosaki, T. (1999) Annu. Rev. Immunol. 17, 555–592
28. Trushin, S. A., Pennington, K. N., Algeiras-Schimnich, A., and Paya, C. V. (1999) J. Biol. Chem. 274, 22923–22931
29. Satterthwaite, A. B., Li, Z., and Witte, O. N. (1998) Semin. Immunol. 10, 309–316
30. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) Nature 401, 82–85
31. Romashkova, J. A., and Makarov, S. S. (1999) Nature 401, 86–90
32. Rawlings, D. J. (1999) Clin. Immunol. 91, 243–253
33. Wang, D., Feng, J., Wen, R., Marine, J. C., Sangster, M. Y., Parganas, E., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J., and Ihle, J. N. (2000) Immunity 13, 25–35
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