Synergistic B Cell Activation by CD40 and the B Cell Antigen Receptor

ROLE OF B LYMPHOCYTE ANTIGEN RECEPTOR-MEDIATED KINASE ACTIVATION AND TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR REGULATION*

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Optimal activation of B-lymphocytes depends both upon expression of various cell surface receptors and adequate integration of signaling pathways. This requires signals generated upon recognition of antigen by the B lymphocyte antigen receptor (BCR) as well as additional signals provided by cognate interaction with T helper cells, including the CD40-CD154 interaction. Engagement of both the BCR and CD40 results in synergistic activation of B cells. Previous studies identified tumour necrosis factor receptor-associated factor (TRAF)-2 and TRAF3 in the CD40-signaling pathway together with BCR-activated protein kinase D (PKD) as important cooperative factors in this synergy. To better understand the role of these factors in bridging the BCR and CD40 signaling pathways, BCR signal regulation of TRAF function was examined. Results show that phosphorylation of TRAF2 is increased upon BCR but not CD40 engagement and that of the potentially phosphorylated residues of TRAF2, tyrosine 484 is crucial for BCR-CD40 synergy. Additionally, wild type or constitutively active Bruton’s tyrosine kinase (Btk) enhanced, whereas the xid mutant form of Btk prevented, BCR-CD40 synergy. These effects were dependent upon TRAF2 and PKD activity. These findings suggest a model in which Btk contributes to the enhancement of the CD40 response by TRAF2 in a PKD-dependent manner.

Activation of B lymphocytes requires expression and ligation of various cell surface receptors as well as adequate integration of the signals received. B cells are exposed to multiple signals simultaneously. Understanding the molecular interactions between the various signaling pathways is important for a complete appreciation of the mechanisms involved in lymphocyte activation.

B lymphocyte activation relies on signals generated upon recognition of antigen by the B cell antigen receptor (BCR)1 and on additional signals provided by cognate interaction with T helper cells, including CD154 binding to CD40 (for review, see Ref. 1). BCR engagement leads to activation of a myriad of intracellular signaling molecules such as members of the protein kinase C (PKC) family of serine-threonine kinases (1). B-lymphocytes express various isoforms of the PKC family, including PKC-α, -β, -γ, -δ, -ε, -ζ, -η, and -μ, and BCR engagement has been shown to induce their activation (2–4). Protein kinase μ (PKD) is a downstream target of second messengers, primarily diacylglycerol, and represents a novel family of serine/threonine kinases. Previous studies show that PKD is activated by multiple signals (for review, see Ref. 5) including BCR engagement in B-lymphocytes (3, 6, 7), and it has been suggested that PKD negatively regulates BCR signaling by phosphorylating Syk and reducing its ability to phosphorylate phospholipase Cγ (3).

CD40 engagement has been shown to cooperate with BCR signals particularly in the germinal center reaction, where follicular dendritic cells present antigen and T helper cells provide CD154. Additionally, detailed analysis of the phenotypic differences between naïve, germinal center, and memory cells show that CD40 and other tumor necrosis factor receptor family members are up-regulated in germinal center cells compared with naïve B cells (8). Co-engagement of the two receptors results in a synergistic activation of B cells (9, 10). Previous studies from our laboratory identified TRAF2 and TRAF3 in the CD40 signaling pathway and BCR-induced PKD activation as playing important roles in the cross-talk between these receptors (10, 11). To integrate the BCR and CD40 signaling pathways, potential ways in which PKD and TRAF molecules cooperate was examined. Because BCR signaling activates a number of kinases, we hypothesized that BCR-mediated TRAF phosphorylation might be important for the synergy. Site-directed mutagenesis of TRAF2 identified the potentially phosphorylatable tyrosine 484 as a critical residue for the synergy and led us to investigate the potential involvement of Bruton’s tyrosine kinase (Btk), a tyrosine kinase, in this process. Our data showed a role for Btk in the synergy, and this was PKD- and TRAF2-dependent. This work together with previous findings provides a model for BCR-CD40 synergy whereby BCR-mediated activation of Btk and PKD modifies TRAF2 in a manner that allows it to block an inhibitory effect mediated by TRAF3.

EXPERIMENTAL PROCEDURES

Cells and Mice—The mouse B cell line CH12.LX expresses surface IgM specific for phosphatidylcholine, an antigen found on the surface of sheep erythrocytes (sheep red blood cells) (12) and has been well characterized (13). The CH12.LacR subclone used for the inducible expression of stably transfected molecules has been previously described (14). Subclones of CH12.LX lacking TRAF2 were produced by homologous
recombination-based gene targeting and are described in detail elsewhere (15). All B cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 10 μM β-mercaptoethanol, and antibiotics (B cell medium). CBA/CJ and CBA/CJH-Btk−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). T-depleted splenocytes from 8–14-week-old mice were prepared as described (9). Sheep red blood cells were obtained from Elmlirn Biologicals (Iowa City, IA) and used in cultures as a source of antigen at a final concentration of 1%. Chinese hamster ovary cells (K1) expressing mCD154 and human CD154-expressing HI-5 insect cells have been described (15, 16).

DNA Constructs and Transfection—The human CD40 (hCD40) construct (17) and PKD constructs have been previously described (11). Point mutations in the TRAF2 molecule were introduced by PCR mutagenesis. All constructs were FLAG-tagged at the COOH terminus and subcloned into the inducible vector pOPRSVI.mcs1 (14). Btk cDNA constructs (WT, R28C, and E41K) were generous gifts from Dr. D. Rawlings (University of Washington, Seattle, WA). All Btk constructs were subcloned into the inducible expression vector pOPRSVI.mcs1 (14). Stable transfections of CH12.LX were performed by electroporation as described (17).

Antibodies and Chemicals—Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Amresco (Solon, OH) and was used at a final concentration of 100 μM. Mouse IgG1 isotype control monoclonal antibody (Ab) (MOPC-21) was purchased from Sigma-Aldrich. Rabbit anti-PKD, rabbit anti-phospho-744/748 PKD, and rabbit anti-phospho Btk (Tyr-223) Abs were purchased from Cell Signaling (Beverly, MA). Rabbit anti-Btk (M-138) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit horseradish peroxidase was purchased from Bio-Rad. Hamster anti-mCD40 (HM40–3) and hamster isotype control monoclonal Ab were purchased from Pharmingen (BD Biosciences). The following Abs were produced in our laboratory by hybridomas purchased from the American Type Culture Collection (Manassas, VA) or provided by the indicated individuals; anti-hCD40 (G28–5, mlgG1) was from ATCC, anti-mouse CD40 (1C10, rat IgG2a) was from Dr. Frances Lund (Trudeau Institute, Saranac Lake, NY), mouse anti-IgE (EM95.3, isotype control, rat IgG2a) was from Dr. Thomas Waldschmidt (University of Iowa, Iowa City, IA). Goat anti-mouse μ-chain specific F(ab′)2 was purchased from Jackson Immuno Research Laboratories (West Grove, PA). Mouse anti-FLAG (M2) antibody was purchased from Sigma-Aldrich. G9676 was purchased from Calbiochem.

**Assays for B Cell Function—** IgM-secreting cells per million recovered viable cells were measured at 72 h of incubation by quantitation on a flow of sheep red blood cells using a direct plaque assay as previously described (18). Inducible PKD, Btk, or TRAF expression was initiated by the addition of IPTG to cultures 18 h before the addition of stimuli. All stimulations proceeded for a total of 72 h, including the time of IPTG incubation.

For proliferation studies 1 × 10^5 cells were stimulated with 1 μg/ml anti-CD40 Ab and/or anti-mCD40 Ab for 48 h with various stimuli. Anti-CD40 monoclonal Abs could not be used as stimuli in this assay because previous studies show that IL-6 production, unlike IgM secretion, requires CD40 stimulation by membrane-bound CD154 (16). IL-6 ELISA was performed as described previously (16).

**Western Blotting and Immunoprecipitation—** CH12.LX cells (5 × 10^5) were stimulated with 10 μg/ml anti-μ-chain specific F(ab′)2 and/or mCD40 for the indicated times. Cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.02% NaN3, and protease and phosphatase inhibitors) for 30 min. The supernatants were incubated with protein G beads preconjugated with appropriate antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, and Western blotting was performed.

**Immunoprecipitation of CH-Labeled Lysates—CH12.LX cells lacking TRAF2 or inducibly expressing FLAG-tagged WT-TRAF2 were labeled as previously described (16).** Cells were stimulated with 10 μg/ml goat anti-mouse μ-chain specific F(ab′)2 and/or mCD154, expressing HI-5 cells for 15 min. Cells were lysed, and TRAF2 was immunoprecipitated. Autoradiography was performed to detect TRAF2.

**RESULTS**

**TRAF2 Dependence of PKD Enhancement of the CD40 Response—** Our previous work has shown that BCR and CD40 synergeste in B cell activation in a TRAF2-dependent manner (10). A mutant hCD40 molecule, hCD40T234A, that binds TRAF3 normally but shows reduced binding to TRAF2 fails to synergize with BCR signals (10, 17). Using pharmacological PKD inhibitors and a dominant-negative PKD molecule to block PKD activity, we also identified PKD as a necessary factor in the BCR-CD40 synergy (11). Collectively these data support the hypothesis that PKD activation may cooperate with TRAF2 to promote synergy between the BCR and CD40. To test this hypothesis, we stably expressed hCD40T234A in CH12.LX cells that can be induced (upon IPTG treatment) to express a constitutively active PKD (PKD-PHD-EE) and asked whether induced expression of this kinase could overcome the synergy defect of the hCD40T234A molecule. We have also previously shown that the transfected wild-type hCD40 and endogenous mCD40 signal indistinguishably in our B cell lines (17). As indicated in Fig. 1A, mCD40 synergizes with BCR in IgM secretion, and as previously shown (10, 17), hCD40T234A does not synergize. Induced expression of PKD-PHD-EE substituted for the BCR signal in synergy with the WT endogenous mCD40. In contrast, PKD-PHD-EE expression was insufficient to replace the BCR signal in cooperation with hCD40T234A.
BCR Mediated TRAF Regulation in BCR-CD40 Synergy

Thus, PKD enhancement of the CD40 response is dependent upon the binding of TRAF2 to CD40.

BCR Engagement and TRAF2 Phosphorylation—These studies support a critical role for a PKD-TRAF2 relationship in integration of the BCR and CD40 signals. We hypothesized that BCR-induced PKD activation might lead to modifications of TRAF2, which are important for the synergy. We focused our attention initially on the possible phosphorylation of TRAF2 after BCR and/or CD40 engagement. To examine the phosphorylation status of TRAF2, cells expressing inducible FLAG-tagged WT TRAF2 were labeled with [32P]orthophosphate. The tagged construct was used because commercially available anti-mouse TRAF2 antibodies do not efficiently immunoprecipitate the protein. Analysis of immunoprecipitated TRAF2 from lysates of cells receiving various stimuli (Fig. 1B) show that TRAF2 was constitutively phosphorylated in B cells and that BCR engagement alone or in combination with anti-CD40 enhanced the phosphorylation of TRAF2. Interestingly, CD40 stimulation did not affect the phosphorylation status of TRAF2.

Role of Conserved Serines in the CD40 Binding Site of TRAF2 in BCR-CD40 Synergy—To address the potential importance of TRAF2 phosphorylation in the synergy between BCR and CD40, we expressed a serine residue mutant of TRAF2 in TRAF2−/− CH12.LX B cells. As previously reported (15), reconstitution of TRAF2-deficient B cells with WT TRAF2 restores the synergy between BCR and CD40. If phosphorylation of TRAF2 is critical for synergy, mutation of potentially phosphorylatable residues to nonphosphorylatable residues would abrogate this function. We used a computer program that predicts possible phosphorylatable sites (19) to identify potential candidate residues. Site-directed mutagenesis was used to construct a TRAF2 molecule in which serines 453 and 455 in the TRAF2 cytoplasmic domain were changed to alanines. These residues were selected on the basis of a predicted high probability that they could be phosphorylated, and both are highly conserved between various TRAFs and among TRAF2 molecules of different species (Fig. 2A). In addition, these residues have been previously shown to be important in binding to CD40 by forming a “serine-tong” surrounding the CD40 residue Gln-232 (20).

The SSAA-TRAF2 mutant was inducibly and stably expressed in TRAF2−/− B cells at levels similar to endogenous TRAF2, and its ability to restore synergy was examined. The SSAA-TRAF2 mutant was able to restore BCR-CD40 synergy in a similar fashion to WT TRAF2 (Fig. 2B). Thus, these serines do not play a significant role in synergy. Interestingly, the binding of this TRAF2 mutant to CD40 was not affected (Fig. 2C), as might have been predicted by previous studies (20), suggesting that the other “hot spots” may compensate in binding of TRAF2 to CD40 in B cells.

Requirement for Tyrosine 484 of TRAF2 for BCR-CD40 Synergy—A TRAF2 molecule in which tyrosine 484 was mutated to phenylalanine was also constructed. Although PKD is a serine/threonine kinase, BCR signaling also leads to activation of a number of tyrosine kinases. Tyr-484 also has a very high probability of being phosphorylated (R = 0.930) and is highly conserved among TRAF molecules in mouse and human (Fig. 3A). The Y484F-TRAF2 mutant was stably and inducibly expressed in TRAF2−/− CH12.LX cells, and its ability to restore synergy was examined. Interestingly, although Y484F-TRAF2 was able to restore the CD40 response, it was not able to restore the synergy between BCR and CD40 (Fig. 3B).

Upon CD40 engagement B cells produce TNF-α, which contributes to IgM secretion via engagement of CD120b/TNFR2 (21). Both CD40 and CD120b associate with TRAF2, so it was possible that the observed defect was due to the inability of CD120b to utilize the Y484F-TRAF2 molecule. This was tested by stimulating cells with TNF-α and measuring the IgM response. As seen in Fig. 3C, the TNF-α response in cells expressing the Y484F-TRAF2 molecule is similar to that of the cells expressing WT-TRAF2, demonstrating that this mutation does not affect all TRAF2 effector functions.

An alternate explanation for the failure of Y484F-TRAF2 to restore synergy could be inability to bind to CD40. It has previously been shown that binding of TRAF2 to CD40 is necessary for CD40-induced degradation of TRAF2 (22) as well as that of TRAF3 (15). The ability of Y484F-TRAF2 to undergo CD40-induced degradation was, thus, examined. As indicated in Fig. 3D, B cells expressing TRAF2 Y484F as the only TRAF2 molecule in the cell showed CD40-mediated degradation of both TRAF2 and TRAF3. However, the extent of degradation for either TRAF2 or TRAF3 was slightly impaired when compared with cells expressing WT-TRAF2, suggesting a potential involvement of Tyr-484 in this function. Finally, WT or Y484F TRAF2 bound to immunoprecipitated mCD40 to a similar extent (Fig. 3E). Therefore, the Y484F mutation specifically affects the synergy-related function(s) of TRAF2 without inhibiting its ability to bind to CD40, mediate signals generated by the CD120b receptor, or induce CD40-induced TRAF degrada-
tion. In addition, to examine the potential phosphorylation of this residue, we compared the phosphorylation status of WT-TRAF2 and Y484F-TRAF2 using ³²P-labeling and phosphotyrosine immunoblotting. There was no detectable difference in phosphorylation between WT and Y484F-TRAF2 (data not shown), perhaps due to the minor contribution of this residue to the overall phosphorylation status of TRAF2. Overall, these data strongly support a necessary role for tyrosine at position 484 of TRAF2 in the synergy between BCR and CD40 and suggest the involvement of a protein-tyrosine kinase in the synergy.

Role of Btk in BCR-CD40 Synergy—The data presented in Fig. 3 imply the involvement of a protein-tyrosine kinase in the synergy between CD40 and the BCR. BCR engagement leads to
activation of several protein-tyrosine kinases, including Syk, Lyn, and Btk. It has been reported that Btk associates with PKD (23), and PKD activation is diminished in Btk-deficient cells (3), however, the significance of such interaction is not clear. Mice containing the naturally occurring point mutation in the PH domain of Btk (R28C) display the xid phenotype, characterized by a block in B-cell development, impaired signaling, and reduced IgM and IgG3 levels (24). Although BCR engagement leads to Btk activation, the involvement of Btk in the CD40 response is not clear. In some reports the CD40 response is not affected by the xid mutation (25), whereas others report a dependence of CD40 on Btk expression (26, 27). Interestingly, mice lacking both Btk and CD40 (27) or simultaneously containing the xid and CD40 deficiency (28) display a more profound B cell immunodeficiency than either of the single knockouts, suggesting a possible link between these molecules.

We examined Btk activation upon BCR and CD40 engagement in CH12.LX cells. BCR engagement leads to Btk activation accompanied by autophosphorylation at Tyr-223 within the SH3 domain (29). BCR engagement led to Btk activation, whereas anti-CD40 treatment did not (Fig. 4). To address the role of Btk in the synergy between BCR and CD40, we inducibly and stably expressed WT-Btk, the xid dominant negative mutant R28C-Btk, and E41K-Btk, a constitutively active molecule that displays increased phosphorylation on tyrosine residues as well as increased membrane association (30). Induced stable expression of each at similar levels is shown in Fig. 5A. Interestingly, expression of WT-Btk enhanced the CD40-induced IgM secretion (Fig. 5B), and a more pronounced enhancement was observed when the E41K-Btk mutant was expressed (Fig. 5C). These data revealed that Btk regulates in part CD40-induced IgM secretion in CH12.LX B cells, consistent with the phenotype of xid mice (27). Interestingly, neither WT-Btk nor E41K-Btk completely substituted for BCR engagement in synergy between this receptor and CD40. BCR ligation enhanced the CD40 response above that observed with CD40 alone, even in the presence of Btk overexpression, pointing to the existence of additional BCR-induced factors involved in the synergy, such as PKD.

The expression of the xid mutant, R28C, abrogated the synergy between BCR and CD40 without affecting the CD40 response (Fig. 5B). This suggests that the R28C mutation specifically affects the BCR-signaling pathway and the interaction between BCR and CD40 signaling pathways.

Additionally, we examined the contribution of Btk in the synergetic activation of splenic B cells from xid mice and WT mice. BCR and CD40 signals synergized in splenic B cell proliferation of B cells from WT mice (Fig. 6, A and B), as shown previously (9, 10). Confirming previous reports (27), BCR engagement was defective in inducing proliferation of xid B cells (Fig. 6, A and B), whereas the CD40 response was unaffected. Despite the inability of BCR to induce proliferation of xid B cells, coengagement of BCR and CD40 had a synergistic effect in B cell proliferation similar to that observed in WT cells (Fig. 6, A and B). Therefore, the synergy between the two signals is able to overcome the xid-induced activation defect in splenic B cell proliferation. This suggests the possibility that distinct biological outcomes of the BCR-CD40 synergy have different requirements for Btk. We have previously shown that BCR and CD40 also synergize in IL-6 production. In contrast to the proliferation data (Fig. 6, A and B), the xid mutation abrogated the ability of BCR and CD40 to synergize in IL-6 secretion without affecting the response to CD40 in the absence of the BCR (Fig. 6C). As expected, synergy was observed in WT B cells. Thus, the differences between proliferation and IgM secretion data above are not attributable to differences between B cell lines versus freshly isolated B cells. The IL-6 results support the more intriguing possibility that requirements and roles of Btk differ for distinct functions in which BCR and CD40 can cooperate.
Relationship between Btk and PKD in the BCR Enhancement of the CD40 Response—Our data demonstrated that both Btk and PKD play a critical role in the synergy. Btk has been shown to associate with PKD in vitro (23), but the biological consequences of this in vivo are not known. Although BCR engagement leads to activation of both Btk and PKD, it is unclear from the data whether Btk and PKD are interdependent in their effects on BCR-CD40 synergy. In contrast to the constitutively active PHD-PKD mutant (Fig. 1A), E41K-Btk (Fig. 5C) did not substitute for BCR engagement in the synergy with CD40, suggesting that the Btk effect on synergy is PKD-dependent. To test this possibility more directly, B cells were stimulated through CD40 and/or the BCR in the presence or absence of induced expression of Btk E41K with or without a PKD inhibitor. We have previously shown that treatment of cells with Go6976 inhibits PKD activation and abrogates BCR-CD40 synergy (Ref. 11 and Fig. 7). Interestingly, the enhancement of the CD40 response upon Btk E41K expression as well as the additional augmentation via BCR engagement was abrogated upon PKD inactivation via Go6976 treatment of cells. It was unclear...
From these data whether PKD is immediately downstream of Btk and whether Btk activation is necessary for PKD activation. Thus, to test the requirement for Btk in PKD activation we examined BCR-induced PKD activation in cells from WT and xid mice. As shown in Fig. 7B, the xid mutation does not abrogate PKD activation in B cells, demonstrating that Btk is required for CD40-BCR synergy independent of its potential involvement in PKD activation. The inhibitor data support a model in which Btk activation contributes to BCR-CD40 synergy in a PKD-dependent manner. However, the role of Btk is not to directly activate PKD but rather to cooperate with PKD-mediated signals in enhancing BCR-CD40 interactions.

**DISCUSSION**

Before the present study, we demonstrated that BCR-induced PKD activation was a necessary component of synergistic interactions between CD40 and the BCR. Interestingly, the previous findings showed that this role of PKD required the binding of TRAFs 2 and 3 to CD40. The present study addressed how molecules activated by BCR engagement might affect TRAF-dependent CD40 synergy.

The activation of PKD could affect TRAF2 via direct modification or indirectly via interaction with other molecules. One possible modification by kinases is phosphorylation, and we did see enhanced phosphorylation of TRAF2 after BCR signaling. Our results did not support a role for PKD in direct phosphorylation of the most likely candidate residues (Ser-453 and -455) in TRAF2, however. In addition, PKD inhibition via G6976 treatment of cells, although lowering the overall phosphorylation status of the immunoprecipitated proteins, did not abrogate the BCR-induced phosphorylation of TRAF2 (data not shown). These data suggest that the contribution of PKD in the synergy, although ultimately affecting TRAF2, is mediated through modification of an additional molecule. Interestingly, our studies revealed a requirement for another conserved residue, Tyr-484, in CD40-BCR synergy. Although further work is needed to determine the exact role of Tyr-484, it could facilitate the recruitment of additional factors to CD40. Alternatively, its modification could induce the dissociation of a negative regulatory factor.

The finding that Tyr-484 of TRAF2 is critical for synergy led us to investigate the potential involvement of the tyrosine kinase Btk in the cross-talk between the BCR and CD40, because PKD and Btk have been shown to associate in B cells (23), and PKD activation is diminished in Btk-deficient cells (3). Indeed, R282C-Btk, a dominant-negative Btk, abrogated the synergy between BCR and CD40, a phenotype similar to the expression of dominant-negative PKD (11). Our data also showed that the role of Btk in the synergy was PKD-dependent. Inactivation of PKD via pharmacological inhibition abrogated the ability of E41K-Btk (constitutively active) to enhance the CD40 response seen in the absence of inhibitor. The role of Btk in synergy, thus, depends upon activation of PKD, and the data suggest Btk might be involved (directly or indirectly) in PKD activation. However, we did not detect any difference in PKD activation between B cells from WT and xid mice, supporting distinct but complementary roles for Btk and PKD in the synergy between BCR and CD40. Our studies with mouse splenic B cells from xid mice showed that Btk is involved in the synergy observed in IL-6 secretion. Interestingly, synergy in B cell proliferation was not affected in xid cells even though cells were unresponsive to BCR signals alone. These results suggest that proliferation and IL-6 secretion in B cells utilize distinct signaling components. Of particular interest was the finding that co-engagement of BCR and CD40 overcame the dependence of BCR signals on Btk in promoting B cell proliferation. A similar finding was recently reported by Mizuno and Rothstein (31); however, those studies only looked at cells pretreated with anti-CD40. Our data demonstrate that simply co-engagement of BCR and CD40 is sufficient to overcome the BCR dependence on Btk activation in B cell proliferation, but not IL-6 secretion or IgM production. These data are consistent with our previous studies showing that CD40-induced IgM secretion is partially dependent on IL-6 production (22).

The present findings support a novel role for the PKD-TRAF2 relationship in linking the BCR and CD40 signaling pathways and suggest a potential role for tyrosine phosphorylation in regulating TRAF2-mediated effects on CD40 signaling. Additionally, Btk activation is shown to be required for synergy, dependent upon PKD activation. Both BCR and CD40 localize to membrane rafts upon engagement (33, 34). We propose (Fig. 8) that upon co-engagement of BCR and CD40, the respective signaling complexes are brought into proximity, enabling interactions among them. This would allow molecules involved in BCR signaling, such as Btk and PKD, to interact with and modify the CD40 signaling complex, including TRAF molecules. In WT cells interference with Btk or PKD activation and/or TRAF2-binding to CD40 leads to abrogation of synergy. Therefore, the signaling molecules in B lymphocytes, kinases, or adaptor molecules regulate not only distinct signaling pathways but also play a critical role in bridging such pathways. This would allow diverse and discrete cellular responses depending on the combination of signals provided by the microenvironment.

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