

Protein Kinase C β Controls Nuclear Factor κ B Activation in B Cells Through Selective Regulation of the I κ B Kinase α

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Abstract

Activation of the nuclear factor (NF)- κ B transcription complex by signals derived from the surface expressed B cell antigen receptor controls B cell development, survival, and antigenic responses. Activation of NF- κ B is critically dependent on serine phosphorylation of the I κ B protein by the multi-component I κ B kinase (IKK) containing two catalytic subunits (IKK α and IKK β) and one regulatory subunit (IKK γ). Using mice deficient for protein kinase C β (PKC β) we show an essential role of PKC β in the phosphorylation of IKK α and the subsequent activation of NF- κ B in B cells. Defective IKK α phosphorylation correlates with impaired B cell antigen receptor-mediated induction of the pro-survival protein Bcl-xL. Lack of IKK α phosphorylation and defective NF- κ B induction in the absence of PKC β explains the similarity in immunodeficiencies caused by PKC β or IKK α ablation in B cells. Furthermore, the well established functional cooperation between the protein tyrosine kinase Bruton's tyrosine kinase (Btk), which regulates the activity of NF- κ B and PKC β , suggests PKC β as a likely serine/threonine kinase component of the Btk-dependent NF- κ B activating signal transduction chain downstream of the BCR.

Key words: B cell survival • B cell receptor • signal transduction • I κ B kinase complex • Btk

Introduction

Efficient B cell immunity requires mechanisms that guarantee the representation of a diverse immunoglobulin repertoire within the peripheral B cell pool (1). A key feature of peripheral B cells in this context is their ability to persist in the absence of antigenic stimulation. The relatively long life of mature naive B cells in peripheral lymphoid organs of mice and man increases the likelihood for an individual B cell to encounter an antigen and initiate an effective humoral immune response (2). Conversely, reduced peripheral B cell survival is frequently associated with naturally occurring or genetically engineered immunodeficiencies (3, 4).

Survival of peripheral B cells is critically dependent on the surface expression of the B cell antigen receptor (BCR) and its ability to generate a signal. Ablation of surface expressed BCR on peripheral B cells by Cre recombinase-mediated modification of the Ig locus or inducible inactivation of Syk protein kinase gene lead to rapid B cell death

(5; and unpublished data). These observations suggest a BCR-dependent signaling cascade responsible for B cell survival in the absence of antigenic stimulation. Furthermore, reduction in the expression level of Bcl-2 protein after the BCR ablation and the rescue from apoptosis of BCR-deficient B cells by Bcl-2 overexpression point to the existence of a signaling axis connecting the BCR and transcription of anti-apoptotic Bcl-2 family of proteins (5). A likely link between the BCR and expression of the anti-apoptotic proteins is suggested by the ability of the BCR to trigger the activation of nuclear factor (NF)- κ B, which is known to control the expression of Bcl-2 and Bcl-xL (6). Successful activation of NF- κ B and hence transcription of the NF- κ B-dependent genes by various ligands requires the phosphorylation of the inhibitory protein I κ B. Unphosphorylated I κ B is bound to the complex of the transcription activating Rel-subunits and retains these within the cytoplasm. Upon cellular activation, I κ B is phosphorylated on serine residues by the multicomponent I κ B kinase (IKK) containing two catalytic subunits (IKK α and IKK β) and one regulatory subunit (IKK γ). Phosphorylation of I κ B targets it for ubiquitination and subsequent degradation by the proteasome (7, 8).

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Recent studies demonstrated that phosphorylation of I κ B α downstream of BCR triggering is regulated by the Bruton's tyrosine kinase (Btk; references 9 and 10). Mutation of Btk results in X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice (11). BCR cross-linking induces I κ B α phosphorylation in wild-type B cells, whereas Btk-deficient B cells or Xid B cells are unable to support I κ B α phosphorylation and efficient activation of NF- κ B. Combined with the short life-span and inefficient Bcl-xL induction of Btk-deficient/Xid B cells, these data support an important role of Btk-mediated NF- κ B activation in BCR-dependent B cell survival (9, 10, 12).

The substrate specificity of Btk as a tyrosine kinase precludes its direct involvement in the phosphorylation of IKKs or I κ Bs on serine residues. This, in turn, implies the existence of a Btk-dependent serine kinase involved in the regulation of IKK activity, I κ B phosphorylation, and NF- κ B activation (9, 10).

Recently, a functional link between Btk and the Ca²⁺/diacylglycerol (DAG)-dependent serine/threonine protein kinase C β (PKC β) was demonstrated. B cells of mice deficient for PKC β display a signaling phenotype similar to Xid B cells (13–15). We therefore hypothesized that PKC β may be involved in the Btk-mediated NF- κ B activation in B cells.

Here we demonstrate that PKC β selectively controls phosphorylation of IKK α in mature B cells and is required for the efficient BCR-induced I κ B α phosphorylation, degradation, and subsequent NF- κ B induction. Furthermore, impaired phosphorylation of IKK α and compromised activation of NF- κ B correlate with defective BCR-mediated induction of Bcl-xL in B cells, thus providing a mechanistic explanation for the reduced life-span of PKC β -deficient B cells.

Materials and Methods

Mice. PKC β ^{-/-} mice on C57BL/6 or 129/Sv genetic background were used for analysis (13). Mice were kept in the animal facility of the Institute for Genetics at the University of Cologne or in the SPF facility of the Laboratory Animal Research Center at The Rockefeller University following the university guide lines. Wild-type C57BL/6 or 129/Sv mice were purchased from The Jackson Laboratory or Charles River Laboratories, respectively.

B Cell Proliferation. Splenic B cells of >95% purity were isolated as described previously (16). Splenic B cells were stimulated *in vitro* with 1.2 μ g/ml goat anti-mouse IgM F(ab')₂ (Jackson ImmunoResearch Laboratories), 7.5 μ g/ml anti-CD38 (17), or 5.25 μ g/ml anti-RP105 (18) alone or in combination with 25 U/ml recombinant mouse IL-4 (Genzyme) as described previously (16). Labeling of cells with 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes) for measurement of the proliferative responses was performed as described (19). The decline in CFSE fluorescence as measure of B cell proliferation was determined by FACS[®] analysis.

Cell Survival Assay. Purified splenic B cells were cultured in the absence or presence of 25 U/ml recombinant mouse IL-4 (Genzyme) for various times. Cells were washed once with ice-

cold Annexin V binding buffer (10 mM HEPES, pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) and cell pellets were stained with Annexin V (Roche) and 7-aminoactinomycin D (7AAD; Sigma-Aldrich) as described (5).

Western Blotting Analysis and EMSA. The expression level of Bcl-2 and Bcl-xL proteins were analyzed by Western blotting. Splenic B cells were purified as described previously (16). 2 \times 10⁶ purified B cells were cultured with 10 μ g/ml F(ab')₂ fragment goat anti-IgM (Jackson ImmunoResearch Laboratories) for indicated time. Cells were harvested and lysed by 1% NP-40 containing buffer as described previously (16). Anti-Bcl-2 (Neomarkers) and anti-Bcl-xL (Cell Signaling Technology) were used for this analysis. The loading of the protein was controlled by Western blotting of anti- α -Actin (Oncogene Research Products).

For the analysis of Rel family protein expression, the protein lysate of purified splenic B cells from PKC β ^{-/-} and control mice were analyzed by Western blotting with antibodies against RelA, RelB, c-Rel, and p52 (Santa Cruz Biotechnology, Inc.). To analyze the IKK α /IKK β /IKK γ complex, purified splenic B cells were lysed 1% CHAPS containing buffer. After the centrifugation and removal of the insoluble fraction, the protein lysates were precleared by rabbit Ig and protein A-sepharose. The IKK complex was immunoprecipitated with anti-IKK α antibody (Cell Signaling Technology) or control rabbit Ig followed by the incubation with protein A-sepharose. Sepharose beads were washed three times with lysis buffer and subjected to SDS-PAGE and transferred onto PVDF membrane. The membrane was incubated with anti-IKK α (Upstate Biotechnology), anti-IKK β (Upstate Biotechnology), or anti-IKK γ antibodies (Santa Cruz Biotechnology, Inc.).

NF- κ B activation was studied after the stimulation with 20 μ g/ml goat anti-mouse IgM F(ab')₂ (Jackson ImmunoResearch Laboratories) or 10 μ g/ml anti-CD40 (3/23; BD PharMingen) using cytoplasmic and nuclear extracts prepared as described (19). Degradation and phosphorylation of I κ B α protein were measured by Western blotting with anti-I κ B α antibody (Santa Cruz Biotechnology, Inc.) and anti-phospho I κ B α (Ser32; Cell Signaling Technology). The phosphorylation of IKK α and IKK β was analyzed by the antibody against phospho-specific form of IKK α (Ser180) and IKK β (Ser181; Cell Signaling Technology). For the loading control, the membranes were stripped and reprobed with anti-IKK α and anti-IKK β antibodies (Upstate Biotechnology). DNA-binding activity of NF- κ B was analyzed by electrophoretic mobility shift assay (EMSA) as described (19). For the supershift, antibodies against p50, RelA and c-Rel (Santa Cruz Biotechnology, Inc.) were added before the binding reaction with DNA. A mutant oligonucleotide was used to test the specificity of DNA binding. The protein loading was determined by Oct-1 EMSA. All quantifications were done with NIH image software.

Results and Discussion

B cells deficient for Btk or expressing a mutant form of the Btk protein are characterized by drastically reduced life-span *in vitro* and *in vivo* (12). Similar properties are characteristic for PKC β -deficient B cells. Incubation of *in vivo* isolated splenic PKC β -deficient B cells *in vitro* in the absence of exogenously added agonists of B cell survival is accompanied by a rapid decline of cell viability (Fig. 1 A). Similar to B cells with impaired Btk function, the PKC β -deficient B cells are characterized by drastically decreased proliferative responses to the antibody-mediated cross-link-

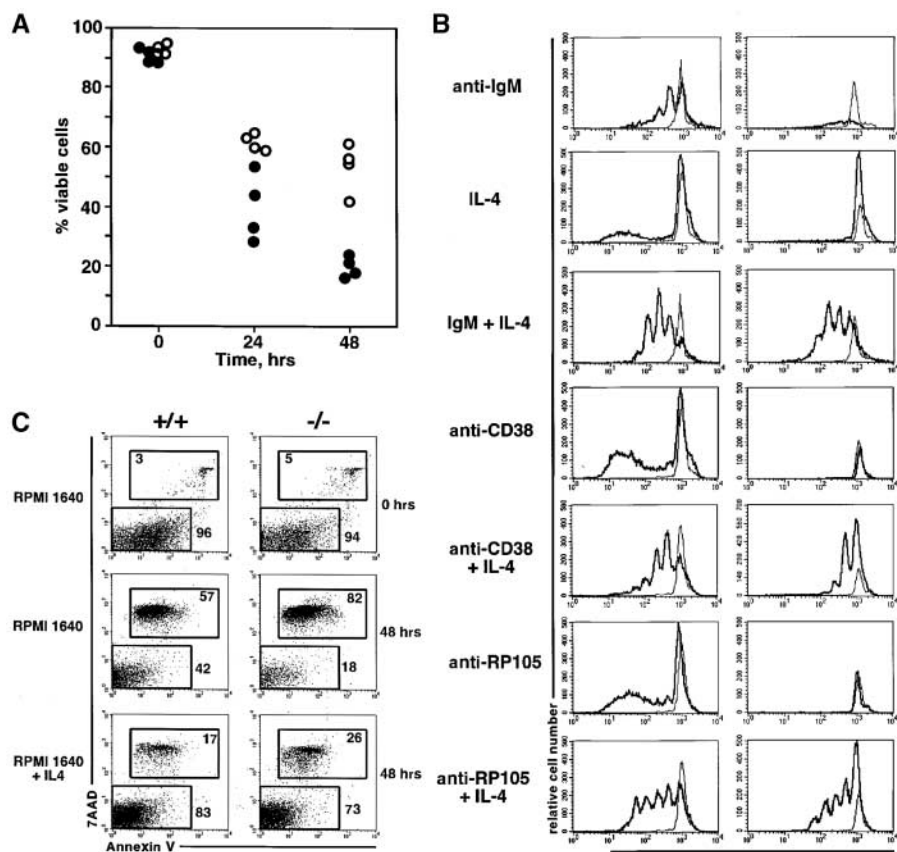


Figure 1. Impaired survival and proliferation of PKCβ-deficient B cells in vitro. (A) Accelerated cell death of PKCβ^{-/-} B cells cultured without stimulation. Percentage of cell viability of purified splenic B cells is plotted for wild-type (129/Sv) control (open circles) and PKCβ^{-/-} (filled circles) B cells. Each dot represents cells isolated from an individual mouse. (B) Impaired proliferative responses of PKCβ^{-/-} B cells in vitro in the absence of IL-4 survival signal. Purified splenic B cells of wild-type (left column) or PKCβ^{-/-} mice (right column) were labeled with CFSE and incubated for 3 d in the presence or absence of the indicated stimuli (see Materials and Methods for details). The thick line indicates the level of CFSE fluorescence in cells upon stimulation, whereas the thin line indicates the CFSE label of nonstimulated cells. Reduction in the CFSE fluorescence is proportional to the number of cell divisions. (C) IL-4 promotes survival of both wild-type and PKCβ^{-/-} B cells. FACS[®] analysis of Annexin-V in combination of 7AAD staining of wild-type (left column) and PKCβ^{-/-} (right column) B cells cultured in complete RPMI with or without IL-4. Note the increase of live cells annexin-V and 7AAD negative in the presence of IL-4. Numbers indicate percentages of gated cells.

ing of BCR, or to the polyclonal activation by anti-CD38 or anti-RP105 antibodies (Fig. 1 B). Addition of the general B cell survival factor IL-4 (20) to the culture medium promotes B cell survival (Fig. 1 C) and increases the fraction of dividing cells (Fig. 1 B). These data suggest that accelerated death of PKCβ-deficient B cells in vitro is likely causing defective proliferative responses of PKCβ-deficient B cells to various stimuli in vitro.

Poor survival of PKCβ-deficient B cells in vitro correlates with their inability to up-regulate expression of the anti-apoptotic protein Bcl-xL upon stimulation with anti-IgM (Fig. 2). Incubation of wild-type splenic B cells with anti-IgM results in progressive increase in the expression levels of the Bcl-xL (Fig. 2). Similar treatment of the

PKCβ-deficient B cells has a negative impact on the Bcl-xL expression level (Fig. 2). The inability of the surface expressed BCR to promote the expression of the anti-apoptotic Bcl-xL and Bcl-2 proteins likely causes the rapid death of the PKCβ-deficient B cells.

Expression of Bcl-xL in B cells is dependent on the activity of the NF-κB transcription factor (6, 21, 22), thus suggesting a possible involvement of PKCβ in NF-κB regulation. Members of the NF-κB/Rel family of proteins include RelA, c-Rel, RelB p50/NF-κB1, and p52/NF-κB2 (7). Deficiency in PKCβ does not affect expression levels of RelA, c-Rel, and RelB in splenic B cells (Fig. 3 A). NF-κB2 is synthesized as a large precursor (p100) that requires proteolytic processing to produce p52 (7). The relative

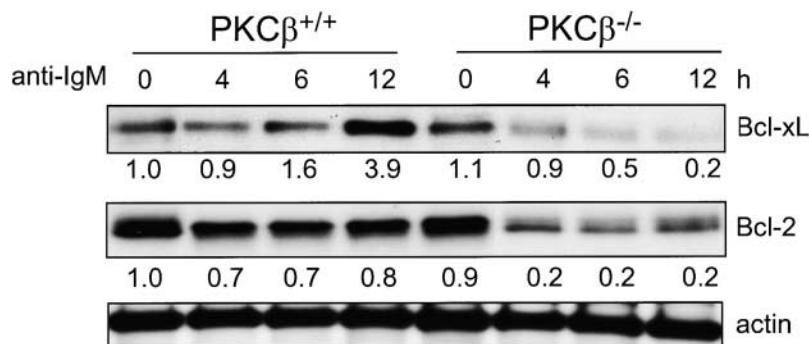


Figure 2. Reduced expression of Bcl-xL and Bcl-2 after IgM cross-linking on B cells of PKCβ^{-/-} compared with control mice. Splenic B cells were isolated from PKCβ^{-/-} and PKCβ^{+/+} mice and incubated with 10 μg/ml anti-IgM for the indicated time (h). Bcl-xL and Bcl-2 protein expression was analyzed by Western blot analysis. Equal protein loading was controlled by anti-actin Western blotting. For quantification, band intensities were first normalized to the respective actin signal and then calculated as fold-change relative to unstimulated PKCβ^{+/+}, which was set to 1.0.

amount of unprocessed p100 NF- κ B2 precursors is increased in PKC β -deficient B cells compared with control B cells (Fig. 3 A).

Defective NF- κ B2 processing has been previously found in B cells deficient for IKK α or expressing a catalytically inactive form of IKK α , which is a component of the trimember I κ B kinase complex IKK α /IKK β /IKK γ (23). To test whether PKC β controls NF- κ B2 processing through the regulation of IKK α expression or activation we have analyzed the expression and activation state of IKKs in PKC β -deficient splenic B cells. Both IKK α and IKK β , as well as the regulatory component of the I κ B kinase complex, IKK γ , are expressed in PKC β -deficient B cells at wild-type levels (Fig. 3 B, WCL lanes). Immunoprecipitation of IKK α or IKK β from PKC β -deficient and control B cell lysates results in coprecipitation of IKK β /IKK γ or IKK α /IKK γ , respectively (Fig. 3 B, and data not shown). These data suggest that expression of IKK α and formation of the I κ B kinase complex is not controlled by PKC β in B cells.

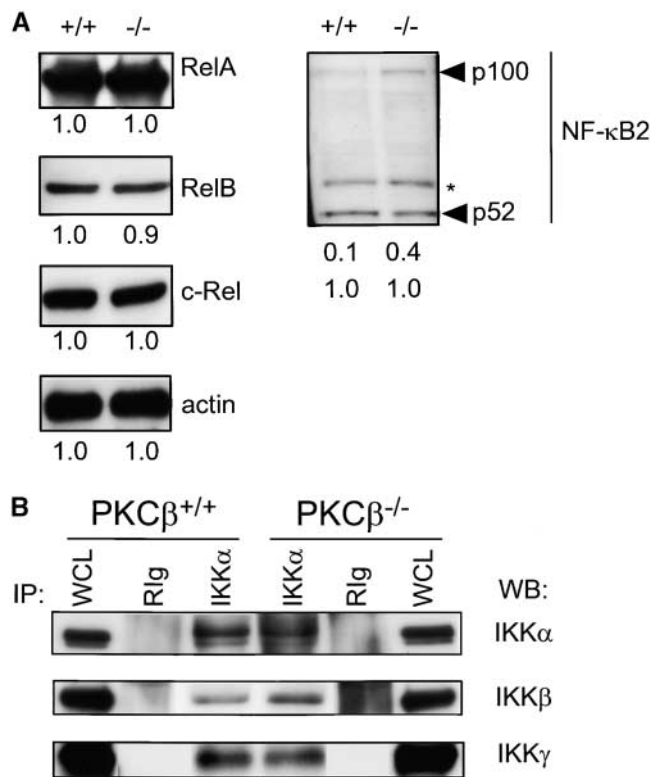


Figure 3. Expression of Rel family proteins and IKK α /IKK β /IKK γ complex in splenic B cells of PKC β ^{-/-} (-/-) and PKC β ^{+/+} (+/+) mice. (A) The amount of RelA, RelB, and c-Rel (left panel) as well as NF- κ B2 (p100 and p52, right panel) were examined by sequential Western blotting. The actin blot (left panel, bottom) was used for quantification as described in Fig. 2. For NF- κ B2 both p100 and p52 are quantified in relation to p52 of +/+ lysates. The asterisk (*) indicates a nonspecific band. (B) Normal composition of the IKK α /IKK β /IKK γ signalosome complex in PKC β ^{-/-} B cells. CHAPS lysates of splenic B cells from PKC β ^{-/-} and PKC β ^{+/+} mice were immunoprecipitated with anti-IKK α antibody (IKK α) or control rabbit immunoglobulin (RIg). Whole cell lysates (WCL) are shown as blotting control. Membranes were sequentially probed with IKK α (top), IKK β (middle), and IKK γ (bottom) antibodies.

Activation of IKK α and IKK β is controlled by their phosphorylation in the activation loop of the kinase domain at serine residues 176/180 and 177/181, respectively (24). Hence, the levels of phospho-IKK α (Ser-180) and phospho-IKK β (Ser-181) reflect indirectly the fraction of activated IKKs within a cell. In wild-type B cells more phospho-IKK α than phospho-IKK β can be detected. Incubation of the wild-type B cells with anti-IgM results in a drastic increase of the amount of phospho-IKK β while the phospho-IKK α levels remain relatively stable in the course of B cell stimulation (Fig. 4 A, top panel). Deficiency in PKC β has a dramatic impact on the phosphorylation state of IKK α and to a lesser extent on IKK β . Phospho-IKK α is virtually absent both in nonstimulated and anti-IgM treated PKC β -deficient B cells (Fig. 4 A, top panel). Moreover, the duration of IgM-mediated phosphorylation of IKK β is diminished in PKC β -deficient B cells compared with the wild-type B cells (Fig. 4 A, top panel). Considering the essential role of serine phosphorylation in IKK α and IKK β activation, these results reveal PKC β as a key regulatory serine/threonine kinase connecting the BCR and IKK activation.

In wild-type B cells, the BCR-mediated activation of IKK α is followed by phosphorylation of I κ B α . This in turn leads to I κ B α degradation and translocation of the Rel proteins to the nucleus. Deficiency in PKC β and ensuing defective IKK α and IKK β phosphorylation result in impaired I κ B α phosphorylation and degradation (Fig. 4 A, middle panel). Congruently, activation of NF- κ B is reduced in PKC β -deficient B cells (Fig. 4 A, bottom panel).

To test whether impaired NF- κ B activation in PKC β -deficient B cells is specific for BCR-mediated signals, we tested stimulation of PKC β -deficient B cells through CD40, which in wild-type B cells also leads to NF- κ B activation. The analysis of IKK α and IKK β phosphorylation in PKC β -deficient B cells stimulated through CD40 reveals a paucity in IKK α phosphorylation similar to that observed after BCR engagement (Fig. 4 B, top panel). However, the CD40-mediated phosphorylation and degradation of I κ B α , as well as the induction of NF- κ B DNA-binding activity, are not impaired in PKC β -deficient B cells (Fig. 4 B, middle and bottom panel). As a member of the TNF receptor family, CD40-mediated NF- κ B activation is thought to require a different set of signaling molecules compared with BCR-mediated NF- κ B activation, namely TRAF2, 3, 5, and 6 (25). Possibly in CD40/TRAF-mediated NF- κ B activation IKK β activation by itself is sufficient, or other kinases, like NIK, can substitute for IKK α (26). In agreement with the normal induction of Rel family proteins CD40 stimulation alone improves the survival of PKC β -deficient B cells to wild-type levels (data not shown). This is also consistent with the finding that the CD40-mediated induction of anti-apoptotic A1 is not impaired in IKK α -deficient B cells (27). In contrast to IgM stimulation, CD40-mediated proliferative responses are only mildly impaired in PKC β -deficient B cells (13), which is also seen in IKK α -deficient B cells (27). In conclusion, the PKC β -mediated regulation of NF- κ B activation is largely specific for BCR-

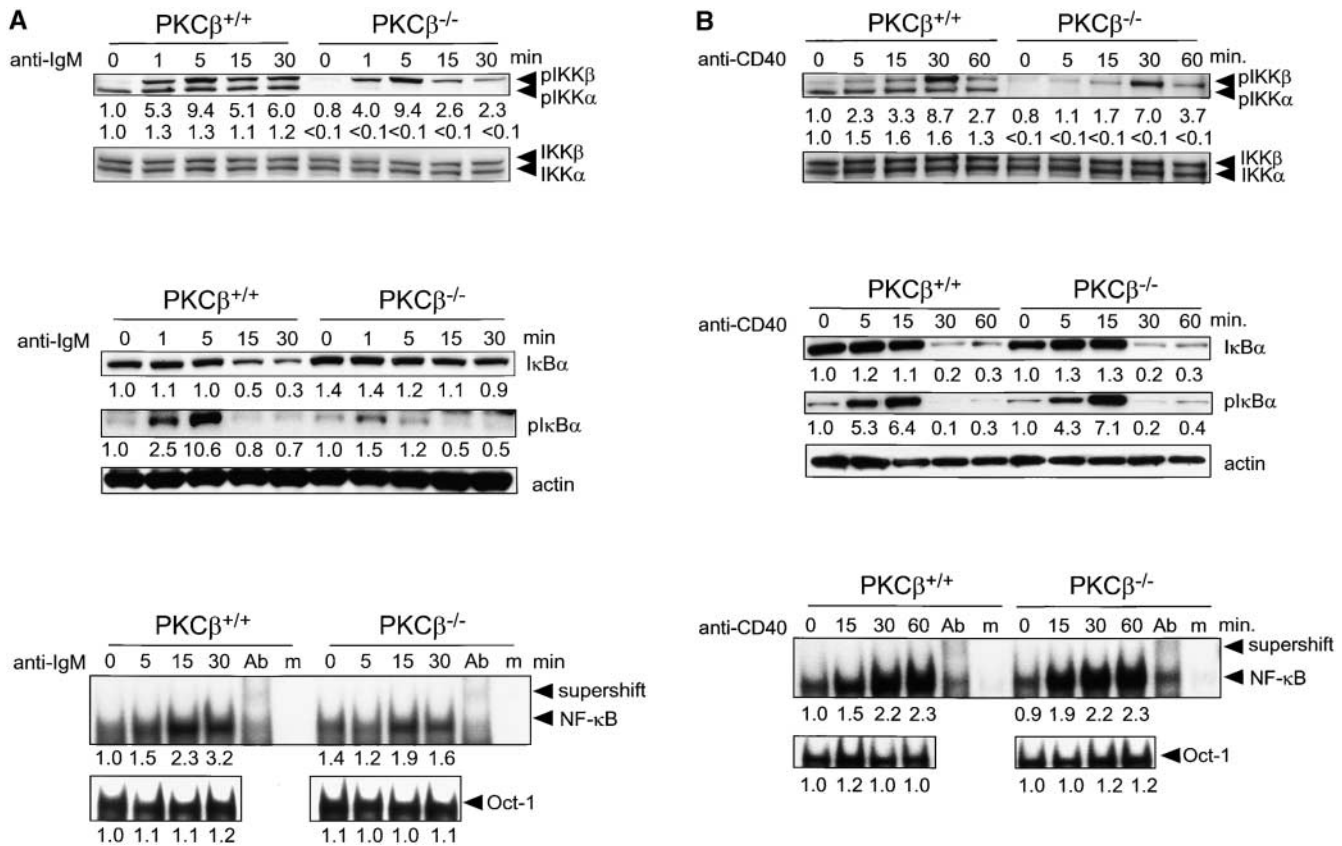


Figure 4. Impaired activation and NF- κ B signaling in PKC $\beta^{-/-}$ B cells after BCR cross-linking. Absence of IKK α activation and shortened IKK β activation in PKC $\beta^{-/-}$ B cells. Splenic B cells of PKC $\beta^{-/-}$ and PKC $\beta^{+/+}$ mice were stimulated with 20 μ g/ml of anti-IgM antibody (A, top panel) or 10 μ g/ml anti-CD40 (B, top panel) for the indicated time (min). Western blot analysis of cytoplasmic extracts were done simultaneously with antibodies specific for phospho-Ser180 and phospho-Ser181 of IKK α and IKK β , respectively (top panel). The membrane was stripped and reprobed with antibodies recognizing nonphosphorylated IKK α and IKK β . For quantification, band intensities of the phosphorylation site-specific blots were first normalized to the respective signal of unphosphorylated protein and then calculated as fold-change relative to unstimulated PKC $\beta^{+/+}$, which was set to 1.0. Inefficient degradation and phosphorylation of I κ B α after IgM cross-linking on PKC $\beta^{-/-}$ B cells (A, middle panel) and no alteration in I κ B α phosphorylation and degradation after CD40 stimulation (B, middle panel). Splenic B cells were treated as in A. I κ B α expression (top) and Ser32 phosphorylation (middle) were analyzed by Western blotting. Equal protein loading was controlled by anti-actin Western blotting (bottom). Quantification was done as in Fig. 2. Reduced DNA binding activity of NF- κ B in PKC $\beta^{-/-}$ B cells after BCR cross-linking (A, bottom panel) and no alteration after CD40 stimulation (B, bottom panel). EMSA was performed with nuclear extracts of splenic B cells treated as in above. The specificity of NF- κ B shift was confirmed by supershift with antibodies for p50, RelA, and c-Rel (Ab) and by using mutant oligonucleotides (m). For quantification, signal intensities were first normalized to the respective signal of an Oct-1 EMSA done with the same nuclear lysates and then calculated as fold-change relative to unstimulated PKC $\beta^{+/+}$, which was set to 1.0.

mediated signaling and has only a minor impact on CD40 mediated B cell activation.

The pattern of defective NF- κ B activation is very similar in B cells of PKC β -deficient and Xid mice (9, 10). As PKC β can act as a negative regulator of Btk, the similar effect of both mutations on NF- κ B activation appears paradoxical. However, increased or decreased Btk-mediated signaling may have the same outcome, as shown by the severe Xid-like phenotype of mice expressing a constitutively active Btk mutant (28, 29). We think that in addition to its inhibitory role for Btk activation, PKC β may also act downstream of Btk to mediate the activation of IKKs. It remains to be seen whether PKC β regulates IKKs through direct phosphorylation. As the activation loop serine residues of IKK α or IKK β are not part of a PKC consensus phosphorylation site, other serine/threonine

residues of IKK α and IKK β may serve as direct substrates for PKC β . Interestingly, a low stringency scan for common PKC phosphorylation sites returns several potential sites for IKK α , but none for IKK β (30). Alternatively, PKC β may regulate IKKs indirectly through an intermediate kinase that would be directly controlled by PKC β . Regardless of the exact mechanism of the PKC β involvement in IKKs phosphorylation, the data presented reveal PKC β as a novel component of the NF- κ B signaling axis responsible for the survival and activation of B cells after BCR cross-linking.

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