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Signal integration by translocation and phosphorylation of PKCδ in the B cell alternate pathway

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Abstract

B cell signaling for activation via the BCR occurs as an isolated event only *in vitro*; in real life, BCR signaling takes place within a complex milieu that involves interactions with agents that trigger additional receptors. Chief among these is IL-4. We have shown that BCR signaling is reprogrammed by IL-4 receptor engagement, and that this reprogramming involves creation of a new, signalosome-independent, Lyn-dependent alternate signaling pathway in B cells isolate from BALB/cByJ mice. A unique aspect of alternate pathway is PKCδ phosphorylation. In dissecting this pathway, we unexpectedly found that Lyn is associated with IL-4Rα, that IL-4 induces Lyn activation, and that Lyn immunoprecipitated from IL-4-treated B cells capably phosphorylates PKCδ in a cell-free system. However, PKCδ phosphorylation doesn't occur in the absence of BCR triggering *in vivo*. This raised the question of why IL-4 alone failed to produce PKCδ phosphorylation. We considered the possibility that Lyn and PKCδ may be spatially separated. As expected, before any treatment, Lyn is located primarily in the membrane fraction, whereas PKCδ is located mainly in the cytosol fraction. However, when anti-Ig follows IL-4 treatment, PKCδ is found in the membrane fraction and phosphorylated. This translocation of PKCδ to the membrane fraction is not affected by loss of Lyn, although PKCδ phosphorylation requires Lyn. Thus, PKCδ phosphorylation through the alternate pathway represents the result of signal integration, whereby neither IL-4 nor anti-Ig working alone produce this outcome, but together achieve this result by Lyn activation (IL-4) and PKCδ translocation (IL-4 followed by anti-Ig).

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Authors Contributions

NK designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. YH and CAL helped design and carry out some experiments, and helped analyze the data. TLR designed the experiments, analyzed the data, and wrote the manuscript.

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Introduction

Activation of quiescent B cells through antigen receptor engagement is essential to the generation of protective serological immunity. As presently understood, multivalent contact with the B cell receptor complex (BCR) triggers a series of biochemical events that result in transcription factor activation leading to a change in the resting program of gene expression (1, 2). Much previous work has shown that surface immunoglobulin (sIg) associated proteins (Igα/CD79a, Igβ/CD79b) direct activation of Src-like kinases that initiate intracellular signaling which propagates through a collection of proximal mediators grouped together as the signalosome, thereby eliciting downstream events that affect gene expression (3). This paradigm, built on extensive biochemical study (here termed the classical pathway) is based mostly on examination of naïve B cells *in vitro*, activated by BCR triggering alone, and thus is not directly applicable to immune system physiology *in vivo*, where B cells do not encounter antigen as an isolated, sterile event but instead integrate multiple receptor-mediated influences before, during and after antigen contact.

Chief among the factors that can influence BCR-triggered B cell stimulation is the pleiotropic cytokine, IL-4. IL-4 is produced by Tfh cells as well as other cell types and has been known for many years to play an important role in B cell physiology by enhancing proliferation induced by BCR engagement, among many other activities (4–11). We began studying the mechanism by which IL-4 affects BCR signaling over 15 years ago and have reported our findings in a series of publications (12–18). We found that prior exposure to IL-4 alters subsequent BCR signaling so that downstream events can proceed in a signalosome-independent fashion. For example, whereas PI-3K inhibition with LY294002 blocks anti-Ig-stimulated ERK phosphorylation, a completely different situation is evident when anti-Ig stimulation follows IL-4 treatment; after IL-4 for 6–24 hours, BCR-triggered ERK phosphorylation is not interrupted by PI-3K inhibition (12). Similar results are obtained with the PLC inhibitor U73122, with the PKC inhibitor Go6976, and with B cells drawn from mice lacking PKCβ (12, 16). In all such cases, anti-Ig stimulation of ERK phosphorylation that is normally blocked by inhibition or deletion of a signalosome element persists when anti-Ig stimulation follows IL-4. In other words, B cell exposure to IL-4 results in creation of a signalosome-independent, alternate pathway for BCR-initiated signal propagation to ERK, whereas IL-4 alone has no effect on ERK phosphorylation (17). However, the IL-4-induced alternate pathway for BCR signaling does not replace the classical pathway but operates in parallel with it (16). Importantly, Lyn is absolutely required for BCR-triggered alternate pathway signaling to pERK, whereas Lyn is dispensable for classical pathway signaling which takes place via other Src-family kinases in its absence (16, 19–21). The conjoint action of IL-4R and BCR in generating alternate pathway signaling represents a form of signal integration within B cells.

In the course of elucidating the range of alternate pathway activity, we found that, after IL-4 treatment, anti-Ig induces tyrosine phosphorylation of the novel PKC, PKCδ (specifically pTry311) whereas anti-Ig treatment alone completely fails to do so (13). Phosphorylation of PKCδ adds a new dimension and further refinement to the alternate pathway because here deletion of signalosome-independent signaling does not rely on a chemical inhibitor or genetic manipulation to quench concurrent signalosome-dependent (classical) signaling.

Since phosphorylation of PKCδ occurs only when BCR triggering follows IL-4 treatment, this phosphorylation is a unique indicator of alternate pathway signaling. Moreover, complete absence of PKCδ does not disturb BCR signaling to pERK in IL-4-treated B cells, indicating that phosphorylation of PKCδ is a separate and distinct branch of non-classical, alternate pathway signal propagation (16). We showed that PKCδ phosphorylation produces activation of PKD and phosphorylation of HDAC5/7, indicating the importance of this branch to molecular events (13). Like the alternate pathway leading to pERK, the alternate pathway leading to pPKCδ absolutely requires Lyn (13). Thus, Lyn appears to act as a gatekeeper that provides the means for BCR signals to travel via the alternate pathway in its various forms.

The unique quality of PKCδ phosphorylation as an outcome of alternate pathway signaling (when anti-Ig follows IL-4), that does not occur during classical pathway signaling (when B cells are stimulated by anti-Ig alone), presented an opportunity to explore how the alternate pathway works. In particular, Lyn and PKCδ are present in naïve B cells in the same amounts as are present after B cells are exposed to IL-4 for 24 hours (13). This raised the question of why PKCδ is not phosphorylated after anti-Ig stimulation alone, considering that the key constituents are readily available, and how PKCδ phosphorylation comes about. In the work described below, we discovered two unexpected elements responsible for PKCδ phosphorylation via the alternate pathway. We found that IL-4 alone induces Lyn phosphorylation and activation. We found that BCR triggering induces PKCδ translocation from the cytosol to the membrane (where Lyn is located), but that this occurs only when anti-Ig follows IL-4. These findings illuminate key mechanistic features of the IL-4-induced alternate pathway for BCR signaling and represent a molecular pathway for intracellular integration of signals derived from separate and distinct receptors.

Material and Methods

Animals:

Eight to fourteen week old male BALB/cByJ and C57BL/6J mice were obtained from The Jackson Laboratory. Lyn-deficient mice on the C57BL/6 background have been described previously (20). Animals were rested and handled in accordance with National Institutes of Health and institutional guidelines, and studies with these mice were approved by the Institutional Animal Care and Use Committee.

B cell purification:

Spleen cell suspensions were prepared from euthanized mice and total B cells were purified by negative selection using magnetic-activated cell sorting (Miltenyi Biotec) according to the manufacturer's instructions.

B cell stimulation:

Purified B cells were cultured at 2 X 10^6 per ml in RPMI 1640 medium supplemented with 5% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME as described previously (12). B cells were treated with IL-4 (20 ng/ml) for different periods of time. B cells were stimulated by $F(ab')_2$ goat anti-mouse IgM at

15 μg/ml (anti-Ig). B cells were stimulated with anti-Ig after incubation in medium alone for 3 h, or after incubation with IL-4 for 24 h, followed by incubation in medium for 3 h. Inhibitors were added 1 h before stimulation with anti-Ig.

Western immunoblot analysis:

Whole cell lysates from B cells were prepared by using NP-40 lysis buffer supplemented with phosphatase (phosphoSTOP) and Complete protease inhibitor cocktails (Roche Inc.). The samples were denatured by boiling at 100°C for 10 min in 1X SDS-PAGE sample buffer supplemented with β-mercaptoethanol. Equal amounts of protein samples were resolved by SDS-PAGE and transferred to PVDF membranes as described previously (12). Immunoreactive proteins were detected by ECL (Amersham Biosciences) using specific antibodies. Immunoblots were stripped and re-probed with control Ab to verify that equal amounts of protein were loaded in each lane.

Immunoprecipitation:

A total of 2 million naive or IL-4-treated B cells were stimulated with 15 μg/ml $F(ab')_2$ fragments of anti-IgM antibody for 10 min, and stimulation was terminated with ice-cold PBS. The cells were collected and lysed with buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml Leupeptin and supplemented with Complete C protease inhibitor cocktail (Roche Inc.). The clear lysates were incubated with anti-Lyn or anti-IL-4Rα antibody with gentle rocking overnight at 4°C. The immunocomplex was further incubated with protein A/G agarose beads (Thermo Scientific) with gentle rocking for 2 hr at 4 ±C. The beads were washed 3 times with lysis buffer and twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$).

Co-immunoprecipitation.

A total of 10 million naive or IL-4-treated B cells were collected and lysed with buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Digitonin, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and supplemented with Complete C protease inhibitor cocktail (Roche Inc.). The clear lysates were directly incubated with protein A/G agarose beads (Thermo Scientific) to immunoprecipitate BCR. To immunoprecipitate IL-4R, the clear lysates were incubated with anti-IL-4Rα antibody with gentle rocking overnight at 4°C. The immunocomplex was further incubated with protein A/G agarose beads with gentle rocking for 2 hr at 4 ±C. The beads were washed 3 times with lysis buffer.

In vitro kinase assay:

Immunoprecipitated Lyn was resuspended in 40 μl kinase buffer supplemented with 200 μM ATP and mixed with recombinant mouse PKCδ (1 μg per reaction; Sino Biological). The reaction mixtures were incubated for 30 min at 30°C. The reactions were quenched with 10 μl 5X SDS sample buffer and vortexed, after which samples were heated to 100°C for 10 minutes. Samples were centrifuged at 300 × g for 30 seconds and supernatants were resolved

on SDS-PAGE. Immunoblotting was performed using phospho-PKCδ (pTyr311) antibody to detect Lyn-mediated phosphorylation of PKCδ. In some experiments, immunoprecipitated Lyn was incubated with ATP and western blotted with anti-pTyr396Lyn.

Subcellular extraction of proteins:

Subcellular extraction of proteins was carried out by using the Subcellular Proteome Extraction Kit (ProteoExtract®; Calbiochem) according to the manufacturer's instructions with minor modifications. Briefly, a total of 20 million unstimulated and anti-Ig stimulated (15 μg/ml for 10 min) naïve and IL-4 treated B cells were harvested and washed with 1 mL ice-cold wash buffer. Pellets were resuspended in 0.5 mL ice-cold Extraction Buffer I supplemented with protease and phosphatase inhibitor cocktails. The mixtures were incubated for 10 min at 4°C on a rotary shaker. Samples were centrifuged at 1000 × g at 4°C for 10 min and the cytosolic protein fractions were collected without disturbing insoluble material. Insoluble material was resuspended in 0.5 ml ice-cold Extraction Buffer II supplemented with protease and phosphatase inhibitor cocktails. The mixtures were incubated for 30 min at 4°C on a rotary shaker. The mixtures were centrifuged at 6000 × g at 4°C for 10 min and the membrane protein fractions were collected without disturbing the insoluble material. Similarly, nuclear proteins and cytoskeleton fractions were enriched with Extraction Buffer III and Extraction Buffer IV; respectively according to the manufacturer's instructions.

Reagents:

Affinity-purified $F(ab')_2$ fragments of polyclonal goat anti-mouse IgM (anti-Ig), and HRP-conjugated goat anti-rabbit secondary antibodies, were obtained from Jackson ImmunoResearch Laboratories. Anti-phospho-PKCδ (pTyr311), anti-PKCδ, anti-phospho-Lyn (pTyr507), anti-Lyn, anti-phospho-Fyn (pTyr530), anti-Fyn, anti-phospho-Blk (pTyr389), anti-Blk, anti-phospho-Syk (pTyr352), anti-Syk, anti-phospho-Src (pTyr416), anti-Src anti-JAK1, anti-JAK3, anti-GAPDH, and anti-actin Abs for immunoblotting were obtained from Cell Signaling Technology. Anti-phospho-Lyn (pTyr396) was obtained from Abcam. Protein A/G ultralink resin was obtained from Thermo Scientific. LY294002, was obtained from Calbiochem. PP2 and PP3 were obtained from abcam. SU6656 was obtained from Santa Cruz Biotechnology. Recombinant murine IL-4 and goat anti-IL-4Rα antibody was obtained from R&D Biosystems. HRP-conjugated rabbit anti-goat secondary antibody, was obtained from southern biotech.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 software (GraphPad Software Inc., USA). The data were analyzed by unpaired t test. p values of < 0.05 were considered statistically significant. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

Results

PKCδ is phosphorylated *in vitro* by Lyn from IL-4-treated B cells

Phosphorylation of PKCδ on tyrosine311 via the alternate pathway, triggered by anti-Ig in IL-4-treated B cells, was blocked by the Src kinase inhibitor, PP2, but not by its

inactive analog, PP3 (Fig 1A). Although not unexpected, this together with the requirement for Lyn, and previous co-IP results, raised the possibility that Lyn itself might directly phosphorylate PKCδ (13). We used *in vitro* kinase assays to test this possibility. We immunoprecipitated Lyn from 4 groups of B cells—B cells incubated in medium for 3 hours (naïve B cells); naïve B cells stimulated with anti-Ig for 10 minutes; B cells incubated with IL-4 for 24 hours followed by medium for 3 hours (IL-4-treated B cells); and IL-4-treated B cells stimulated with anti-Ig for 10 minutes—and tested immunoprecipitated Lyn for its ability to phosphorylate PKCδ in the presence of ATP. As expected, we found that Lyn immunoprecipitated from IL-4-treated B cells stimulated with anti-Ig (anti-Ig/IL-4) phosphorylated PKCδ whereas Lyn immunoprecipitated from naïve B cells, and naïve B cells stimulated with anti-Ig, failed to phosphorylate PKCδ (Fig 1B). We also found, most unexpectedly, that Lyn immunoprecipitated from IL-4-treated B cells, that had not been stimulated with anti-Ig, phosphorylated PKCδ to a similar extent as Lyn from IL-4-treated B cells that had been stimulated with anti-Ig. In other words, B cell treatment with IL-4 was all that was needed (and BCR triggering was not needed) for Lyn to acquire the ability to phosphorylate PKCδ *in vitro* in the absence of any other cellular constituents. However, this contrasts with the situation in whole cells, wherein PKCδ phosphorylation occurs when IL-4 treated B cells are stimulated with anti-Ig, but not when B cells are treated with IL-4 alone or anti-Ig alone (Fig 1C). This unexpected result raised the question of how IL-4 alone induces competence for Lyn kinase to phosphorylate PKCδ *in vitro*, but not *in vivo* in intact B cells.

Lyn is phosphorylated by B cell exposure to IL-4

IL-4-induced Lyn competence for PKCδ phosphorylation *in vitro* could simply reflect Lyn activation resulting from tyrosine 396 phosphorylation. To address this possibility, we treated freshly purified B cells with IL-4, prepared lysates, and then separated proteins by PAGE followed by western blotting for Lyn(pTyr396). We found that IL-4 treatment produced abundant phosphorylation of Lyn(pTyr396) with minimal phosphorylation of the Lyn inhibitory tyrosine at 507 (Fig 2A–D). Having established a connection between IL-4 and pLyn, we examined the speed with which that connection is made. In time course experiments we showed that IL-4-induced Lyn phosphorylation is evident within 15 minutes of B cell exposure to IL-4 (Fig 2E and F). This rapid IL-4-induced Lyn phosphorylation does not appear to involve interaction with the BCR as evidenced by co-immunoprecipitation experiments in which we found no evidence for co-localization of IL-4R and BCR before or after IL-4 treatment (Supplementary Fig. 1). In keeping with this rapid Lyn phosphorylation, exposure of B cells to IL-4 for as little as 1 hour is sufficient to set in motion events that lead to PKCδ phosphorylation after anti-Ig stimulation (Supplementary Fig. 2). Importantly, Lyn phosphorylation induced by IL-4 is selective, in that B cell tyrosine kinases including Syk, Src and other Src-family kinases, including Fyn and Blk, are not phosphorylated by B cell treatment with IL-4 for periods up to 24 hours (Supplementary Fig. 3), although phosphorylation of Syk was increased to a small but significant extent. It is notable that the levels of Lyn, Fyn and Blk are not changed by B cell treatment with IL-4 (Fig. 2D,E,F and Supplementary Fig. 3F,G,H). However, we observed some downregulation in Syk (Supplementary Fig. 3A, C). This observation is consistent with previously published data (37). The short time frame for induction of Lyn(pTyr396) by IL-4 suggested that

Lyn may autophosphorylate following IL-4R activation. To test this notion, B cells were treated with IL-4 after which Lyn was immunoprecipitated, incubated with ATP, subjected to PAGE and western blotted with anti-Lyn(pTyr396). This *in vitro* kinase assay showed B cell treatment with IL-4 produced Lyn autophosphorylation (Fig 3A, B). Further probing showed that immunoprecipitated Lyn was not accompanied by JAK1 or JAK3, and IL-4-induced Lyn phosphorylation was blocked by src kinase inhibitors (Fig 3A, B). Consistent with this, we found that Lyn is associated with immunoprecipitated IL-4Rα, and IL-4Rα is associated with immunoprecipitated Lyn, before (and after) B cell treatment with IL-4 (Fig 3C, D). Although other participants cannot be ruled out, this suggests that a major source of phosphorylated Lyn lies in IL-4R-induced Lyn phosphorylation of itself. Overall, these results present the novel finding that IL-4 alone activates Lyn in B cells by rapidly inducing tyrosine 396 phosphorylation, at least in part via autophosphorylation. This IL-4 activated Lyn is capable of phosphorylating PKCδ *in vitro*; however, this does not occur in intact B cells unless IL-4 is followed by BCR triggering. Notably, the levels of Lyn and PKCδ are similar in unstimulated and IL-4-stimulated B cells.

PKCδ translocates and is phosphorylated after B cell treatment with IL-4 and anti-Ig

PKCδ has been reported to reside in various subcellular compartments, most prominently within the cytosol fraction, and to translocate among them under various stimulatory conditions (22–25), whereas Lyn resides in the membrane compartment (26, 27). We considered the possibility that location and translocation may constitute key elements in Lyn-mediated PKCδ phosphorylation. That is, we evaluated the possibility that activated kinase (Lyn) and its target (PKCδ), which react when brought together *in vitro*, are initially separated *in vivo* and only come together under a specific set of circumstances. To address this, we examined the location and phosphorylation of Lyn and PKCδ in naïve and IL-4-treated B cells, with and without subsequent anti-Ig stimulation.

We prepared cytosol and membrane protein extracts from 4 groups of B cells—B cells incubated in medium for 3 hours (naïve B cells); naïve B cells stimulated with anti-Ig for 10 minutes; B cells incubated with IL-4 for 24 hours followed by medium for 3 hours (IL-4-treated B cells); and IL-4-treated B cells stimulated with anti-Ig for 10 minutes —and examined Lyn, pLyn, PKCδ and PKCδ(pTyr311) by western blotting, along with GAPDH and CD79b as fractionation controls. We showed that fractions were well separated —GAPDH was completely localized to the cytosol fraction and there was no detectable GAPDH in the membrane fraction; CD79b was localized to the membrane fraction and there was vanishingly little CD79b in the cytosol fraction. As previously reported, Lyn and PKCδ reside in different compartments in naïve B cells, with Lyn located in the membrane fraction and PKCδ located in the cytosol fraction (Fig 4). For the most part, Lyn remained in the membrane, and intracellular PKCδ remained in the cytosol, regardless of B cell treatment with anti-Ig, IL-4, or IL-4 followed by anti-Ig. Importantly, the latter two conditions led to changes in these two constituents (Fig 4A). IL-4 treatment, with or without subsequent anti-Ig stimulation, resulted in substantial phosphorylation of Lyn activation Tyr396, without phosphorylation of Lyn inhibitory Tyr507, in the membrane. Changes in PKCδ were perhaps more consequential. Anti-Ig stimulation of naïve B cells had no effect on PKCδ location or phosphorylation, as might be expected in view of our

data indicating that BCR-triggered classical pathway signaling does not result in pPKCδ. However, anti-Ig stimulation of IL-4-treated B cells resulted in the appearance of PKCδ and pPKCδ in the membrane. In other words, when BCR triggering follows IL-4, and only when it follows IL-4, some portion of PKCδ translocates to the membrane where it becomes phosphorylated, presumably by pLyn. Of note, separation of IL-4 and anti-Ig is not required for PKCδ phosphorylation inasmuch as this occurs equally well when IL-4-exposed B cells are not washed prior to anti-Ig stimulation, although long term treatment with both together is not effective (Supplementary Fig. 4). In sum, the translocation of a portion of PKCδ from cytosol to membrane then places activated Lyn kinase and PKCδ substrate in the same compartment, which may be thought of as mimicking *in vitro* phosphorylation of PKCδ by activated Lyn immunoprecipitated from IL-4-treated B cells, in which case these two constituents are artificially and physically forced together.

We further prepared cytosol and membrane protein extracts from 4 groups of B cells as above--naïve B cells, naïve B cells stimulated with anti-Ig, IL-4-treated B cells, and IL-4-treated B cells stimulated with anti-Ig--that were incubated with the PI-3K inhibitor LY294002 for 1 h before anti-Ig stimulation. We found that incubation of B cells with LY294002 before anti-Ig stimulation did not prevent membrane translocation and phosphorylation of PKCδ through the alternate pathway (Fig 4B, C). These data indicate that translocation and phosphorylation of PKCδ are PI-3K independent processes, like other aspects of alternate pathway signaling.

PKCδ translocation is independent of Lyn

These experiments do not rule out the possibility that PKCδ translocates after it becomes phosphorylated, rather than before, which could occur if some other kinase acts instead of, or in addition to, Lyn. To test this, we repeated the experimental conditions described above with splenic B cells obtained from Lyn KO mice, examining the location and phosphorylation of PKCδ. WT B cells for control conditions were obtained from C57BL/6 mice, the strain background for Lyn KO mice. As above, PKCδ was primarily located in the cytosol in naïve B cells and under all conditions of stimulation for B cells obtained from both WT and Lyn KO mice. Further, anti-Ig stimulation of IL-4-treated WT B cells resulted in the appearance of both PKCδ and pPKCδ in the membrane, as expected (Fig 5A). Results in Lyn-deficient B cells were revealing: anti-Ig stimulation of IL-4-treated Lyn KO B cells resulted in the appearance of PKCδ in the membrane, in the complete absence of phosphorylated PKCδ (Fig 5B). In other words, Lyn-deficiency separates and distinguishes PKCδ translocation from PKCδ phosphorylation. The results with Lyn KO B cells show that non-phosphorylated PKCδ is competent to translocate from cytosol to membrane, that PKCδ translocation to the membrane does not require Lyn, and that once in the membrane PKCδ is not phosphorylated in the absence of Lyn.

Discussion

Alternate pathway signaling for PKCδ phosphorylation, which does not occur in classical pathway signaling, has important implications for B cells because events downstream of PKCδ include activation of PKD and HDAC5/7 (13). The present work describes two

novel findings that elucidate the steps leading to pPKCδ. First, we have shown that B cell treatment with IL-4 alone rapidly produces tyrosine phosphorylation and activation of the Src-family kinase, Lyn, which is fully capable of phosphorylating Tyr311 on PKCδ. This IL-4-induced Lyn phosphorylation involves autophosphorylation, and occurs in a specific manner in which other Src family kinases are not similarly phosphorylated. Second, we have shown Lyn kinase phosphorylation of PKCδ is regulated by substrate translocation, which occurs when B cells treated with IL-4 are stimulated with anti-Ig. Such stimulated PKCδ translocation takes place in the complete absence of Lyn; and, Lyn activation without PKCδ translocation, as occurs after B cell exposure to IL-4 alone, fails to phosphorylate PKCδ. Thus, we describe a mechanism in which translocation of a specific substrate is the key event in the pathway leading to its phosphorylation and integrates signals emerging from two separate and distinct surface receptors. This may be a general mechanism for signal integration and receptor crosstalk, which may be a way of regulating the pace and/or intensity of protein interactions involved in signaling cascades or other enzyme:substrate events.

IL-4 has been known for some years to affect B cells and, in particular, to enhance some outcomes of BCR engagement. We have now shown that IL-4 induces activation of Lyn, which to our knowledge has not been previously described. Although Lyn is the most abundant Src-like kinase in B cells (28–31), Lowell and colleagues showed years ago that signaling via the classical, signalosome-dependent pathway occurs more or less normally in the absence of Lyn (19–21). In contrast, all aspects of alternate pathway signaling, including signaling for pPKCδ and pERK, are abolished in Lyn-deficient B cells and thus require Lyn (13, 14, 16). This requirement raises the question of selectivity in Src-family kinase activation and/or activity. Although the similarity among Src family kinases might suggest that any B cell Src-family kinase could phosphorylate any given target, and Fyn has been shown to phosphorylate PKCδ in a non-B cell type (32), our results suggest that other Src-family kinases cannot fulfill the role of activated Lyn kinase in the alternate pathway because other Src-family kinases fail to be activated by IL-4R engagement. A previous report on Lyn and Fyn provides a precedent for this, in that engagement of OX40L selectively suppresses Fyn activity but not Lyn activity, through recruitment of Csk leading to C-terminal inhibitory tyrosine phosphorylation (33). This could extend to other cytokines including IL-4 as suggested by the report that Chk (Csk homologous kinase) mRNA and protein is induced by IL-3, IL-4 and GM-CSF in monocytes [34]. Alternatively, as suggested by our results, Lyn may directly associate with IL-4R and then autophosphorylate, or IL-4R-binding may facilitate Jak-mediated phosphorylation. This has not been described in the literature, but it has been pointed out that a region in IL-4Rα is similar to a region in IL-2Rβ that interacts with Src-family kinases (35), and that Lyn associates with the Epo receptor (36). Thus, the overall composition of active Src-family kinases could be altered in B cells that experience IL-4 (as opposed to antigen), leaving Lyn as the principal kinase that is activated to mediate alternate pathway signal propagation, explaining why the alternate pathway depends completely on Lyn whereas Lyn is compensated by other Src family kinases in the classical pathway. This does not, however, rule out the possibility of Lyn substrate preference for PKCδ that is not reproduced by other Src family kinases.

We previously showed that Lyn and PKCδ co-immunoprecipitate in B cells exposed to IL-4 and stimulated by anti-Ig, and that Lyn immunoprecipitated from B cells treated with IL-4 alone is active and fully capable of phosphorylating PKCδ *in vitro*. With active kinase and substrate present in IL-4 treated B cells, these results indicate that some other element precludes PKCδ phosphorylation under these conditions and is responsible for PKCδ phosphorylation when BCR triggering follows IL-4 treatment. The present work has addressed this element that underlies PKCδ phosphorylation in the alternate pathway and has defined a new and critical intermediary step of PKCδ translocation that is independent of Lyn kinase and substrate phosphorylation. However, the means by which PKCδ is mobilized by BCR triggering after IL-4, but not before, remains undefined.

In previous work we examined Lyn-proximal elements, namely surface immunoglobulin (sIg) associated proteins Igα and Igβ, and we unexpectedly found that exposure to IL-4 alone induces marked upregulation of Igβ, and, to a lesser extent, of Igα at the protein level due to enhanced translation (14). This has been confirmed by another group (37). Upregulation of Igα and Igβ is accompanied by increased IgM maturation and a 2–3-fold increase in IgM on the B cell surface. Experiments with the BAL-17 B cell line confirmed that increased surface IgM is associated with increased BCR signaling for downstream events (14). These results raise the possibility that some or all aspects of the alternate pathway consist of elements already present in naïve B cells; whereas these elements require a higher strength of signal to become activated as compared with elements comprising the classical signaling pathway. In this view, the requirement for initial IL-4 to prepare B cells for BCR-triggered PKCδ translocation reflects IL-4 induction of increased Igα/Igβ/sIgM, engagement of which produces increased signal strength that is able to overcome a barrier to engagement of elements and mechanisms that already exist in B cells and are triggered by BCR engagement if/when that triggering provides a sufficiently intense signal. However, the nature of those pre-existing elements responsible for PKCδ translocation remains unclear, and the relatively short period of time required for IL-4 exposure before BCR engagement produces PKCδ translocation weighs against this mechanism.

A previous report suggested that cholecystokinin induces PKCδ translocation from cytosol to membrane in rat pancreatic acinar cells (38). However, translocation of PKCδ in this model was a “single hit” event and separation of translocation from phosphorylation relied solely on a chemical inhibitor. The present study is the first to our knowledge to define a role for IL-4/BCR crosstalk and signal integration in PKCδ translocation. It is also the first to our knowledge to clearly differentiate PKCδ translocation from subsequent phosphorylation, to identify the kinase responsible for PKCδ phosphorylation in B cells, and to show that these two processes of translocation and phosphorylation operate separately in B cells; in these respects, our study is completely novel.

With IL-4R and BCR engagement, two receptor-generated signals acting sequentially are integrated through two distinct mechanisms to phosphorylate PKCδ. This raises the question of what situations might arise in which IL-4 stimulation precedes BCR engagement. It has been reported that serum IL-4 levels are measurable and elevated in cases of acute infection, trauma, and malignancy (39–43). In other words, in some clinical situations, B cells throughout the body may be exposed to IL-4, which would presumably involve

antigen-specific B cells before any antigen stimulation. Thus, it may be speculated that alternate pathway signaling represents a pathway for B cell activation *in extremis*. It may also be speculated that alternate pathway signaling represents a form of B cell memory, inasmuch as IL-4 can be removed yet subsequent BCR engagement still triggers alternate pathway signaling. Thus, the alternate pathway may participate in stimulating B cells in an environment of acute and/or severe illness, or even as a memory of exposure to IL-4.

In sum, PKCδ phosphorylation through the alternate pathway appears to consist of several discrete steps: 1. IL-4 induces Lyn phosphorylation and activation; 2. IL-4 followed by BCR engagement produces translocation of PKCδ from the cytosol to the membrane; 3. Activated Lyn in the membrane phosphorylates PKCδ in the membrane. A diagram illustrating these steps is shown as (Fig 6). Beyond the novel finding of Lyn activation after IL-4, this work identifies PKCδ translocation--only when BCR triggering follows IL-4 as a critical and necessary step in bringing about PKCδ phosphorylation during alternate pathway signaling and a means of integrating separate and distinct receptor signals. This work emphasizes the physiological importance of BCR signaling in the context of IL-4R engagement, in that some events such as PKCδ translocation only occur under conditions of dual receptor activity, and further emphasizes the role of substrate translocation among compartments as a key mechanism for regulating intracellular signaling. Moreover, target translocation for consummation of enzymatic activity, described here, may be a more general occurrence. This work on alternate pathway signaling can serve as a model for further investigation into physiological B cell activation inasmuch as B cells do not encounter antigen as an isolated, sterile event but instead integrate multiple receptor-mediated influences before, during and after antigen contact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research highlights

- IL-4 treatment induces signalosome-independent alternate pathway for BCR signaling
- Lyn gets autophosphorylate after IL-4 treatment in membrane
- PKCδ translocate and phosphorylate by Lyn in membrane through the alternate pathway

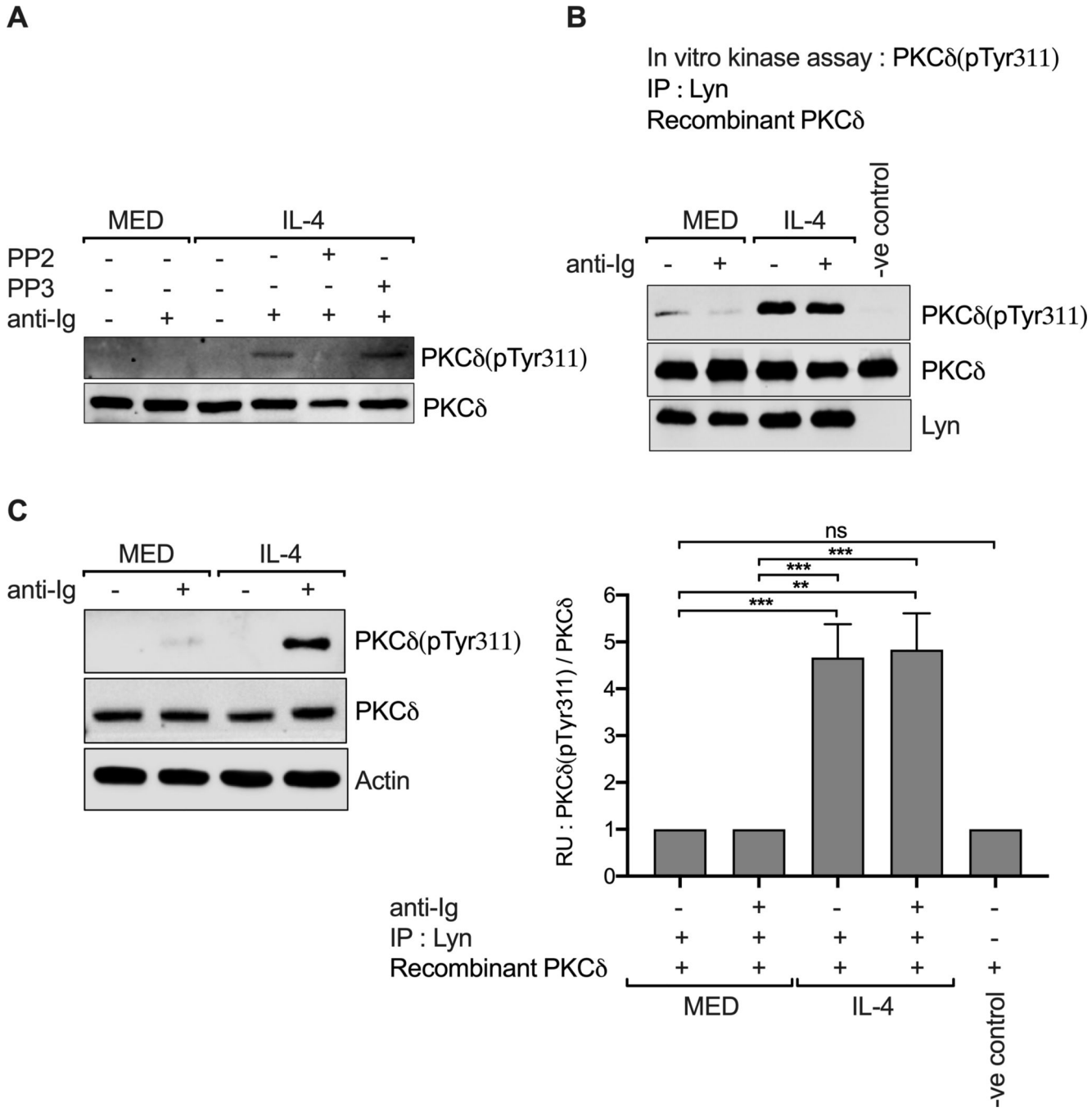

Figure 1: PKCδ is phosphorylated upon BCR stimulation in IL-4-treated B cells but not in untreated B cells.

(**A**) Phosphorylated PKCδ was analyzed by immunoblotting for PKCδ(pTyr311) in whole cell lysates from purified B cells cultured in medium for 3 h (MED) or incubated with IL-4 (20 ng/ml) for 24 h followed by anti-Ig (15 μg/ml) stimulation for 0 (−) or 10 (+) min in the presence or absence of 20 μM PP2 or PP3 (negative control for PP2) for 1 h before anti-Ig stimulation. Membranes were stripped and reprobed with PKCδ-specific Ab as a loading control. (**B**). *In vitro* kinase assays were performed at 30±C for 30 min by

incubating immunoprecipitated Lyn from purified B cells cultured in medium for 3 h (MED) or treated with IL-4 for 24 h followed by anti-Ig stimulation for 0 (−) or 10 (+) min with recombinant PKCδ as substrate. Recombinant PKCδ alone was used as negative control. Immunoblotting with PKCδ(pTyr311)-specific Ab was used to analyze phosphorylation of PKCδ by Lyn. Membranes were stripped and reprobed with PKCδ and Lyn-specific Abs as loading controls. The bar graph shows quantitation of PKCδ(pTyr311) normalized to PKCδ(pTyr311) from medium treated B cells, given a value of 1. (**C**) Purified B cells were cultured in medium for 3 h (MED) or incubated with IL-4 for 24 h followed by anti-Ig stimulation for 0 (−) or 10 (+) min. Whole cell lysates were analyzed by immunoblotting with an antibody directed against PKCδ(pTyr311). Membranes were stripped and reprobed with PKCδ and β-actin specific Abs as loading controls. All experiments were performed at least two to three times with pooled B cells from two to three mice per group in each experiment, and data from a single representative experiment is shown. Values represent mean ± SD. Uppaired t test was used to compare data obtained for MED (with or without anti-Ig) and IL-4 (with or without anti-Ig) treated B cells. p values of < 0.05 were considered statistically significant. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

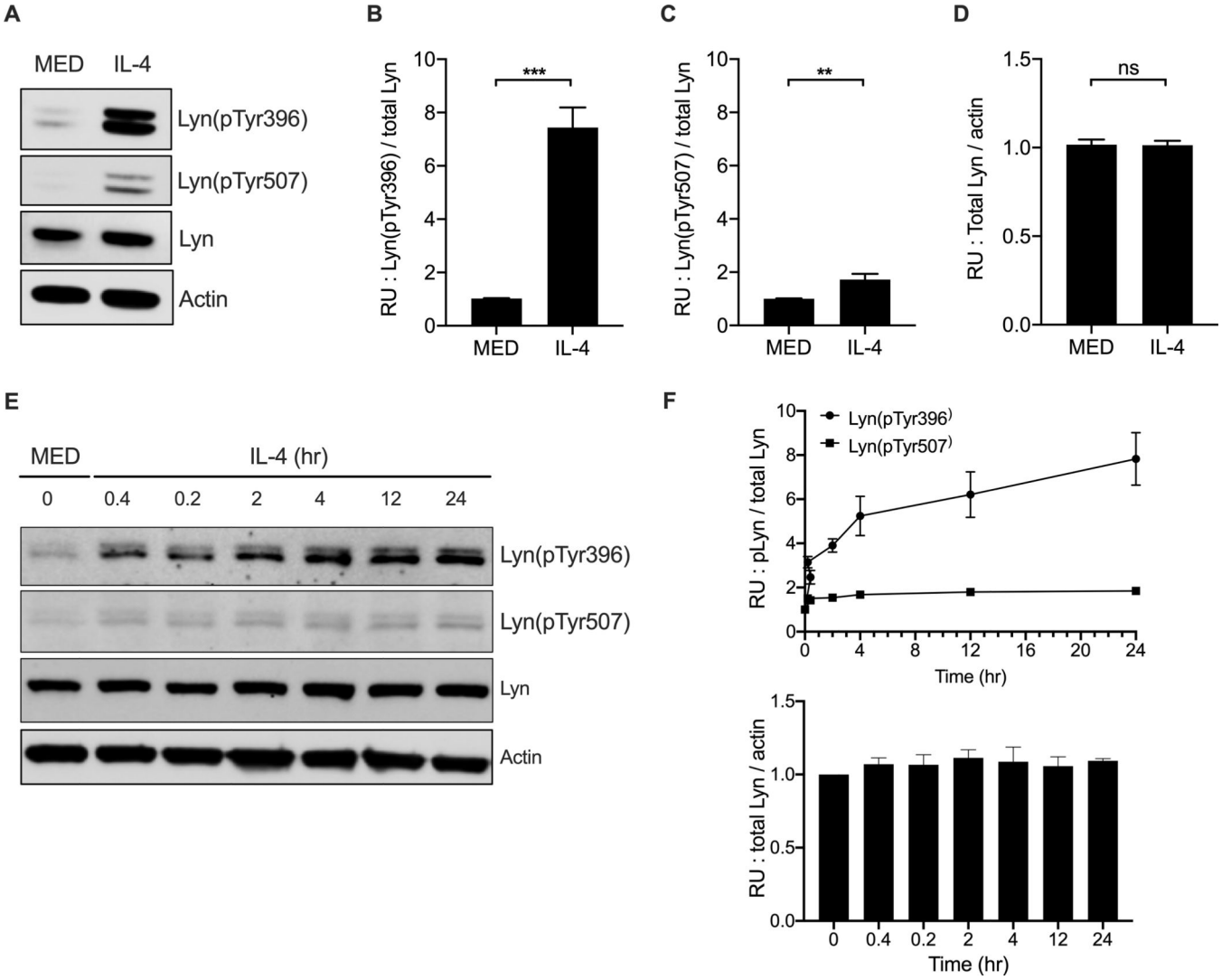

Figure 2: Lyn is phosphorylated in IL-4 treated B-cells.

(**A**) Lyn expression and phosphorylation were analyzed from freshly purified B cells cultured in medium for 3 h (MED) or incubated with IL-4 for 24 h by immunoblotting using Abs directed against Lyn (pTyr396 and pTyr507). Membranes were stripped and reprobed with Lyn and β-actin specific Abs as a loading control. (**B, C, D**) Bar graphs show quantitation of Lyn(pTyr396) and Lyn(pTyr507) in relation to Lyn (**B, C**) and total Lyn in relation to actin (**D**), with values in untreated (MED) B cells set at 1. (**E**) Purified B cells were cultured in medium (MED; 0 min) or treated with IL-4 for the indicated time points, and whole cell lysates were subjected to immunoblotting using Abs directed against Lyn(pTyr396) and Lyn(pTyr507). Membranes were stripped and reprobed with Lyn and β-actin specific Abs as a loading control. (**F**) Line graphs and bar graphs show Lyn(pTyr396) and Lyn(pTyr507) in relation to Lyn (top), and total Lyn in relation to β-actin (bottom), with values for untreated (MED, 0) B cells set at 1. All experiments were performed at least three to four times with pooled B cells from two to three mice per group in each experiment, and data from a single representative experiment is shown in A and E. Values represent mean ±

SD. Unpaired t test was performed to compare the data obtained for MED and IL-4 treated B cells. p values of < 0.05 were considered statistically significant. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

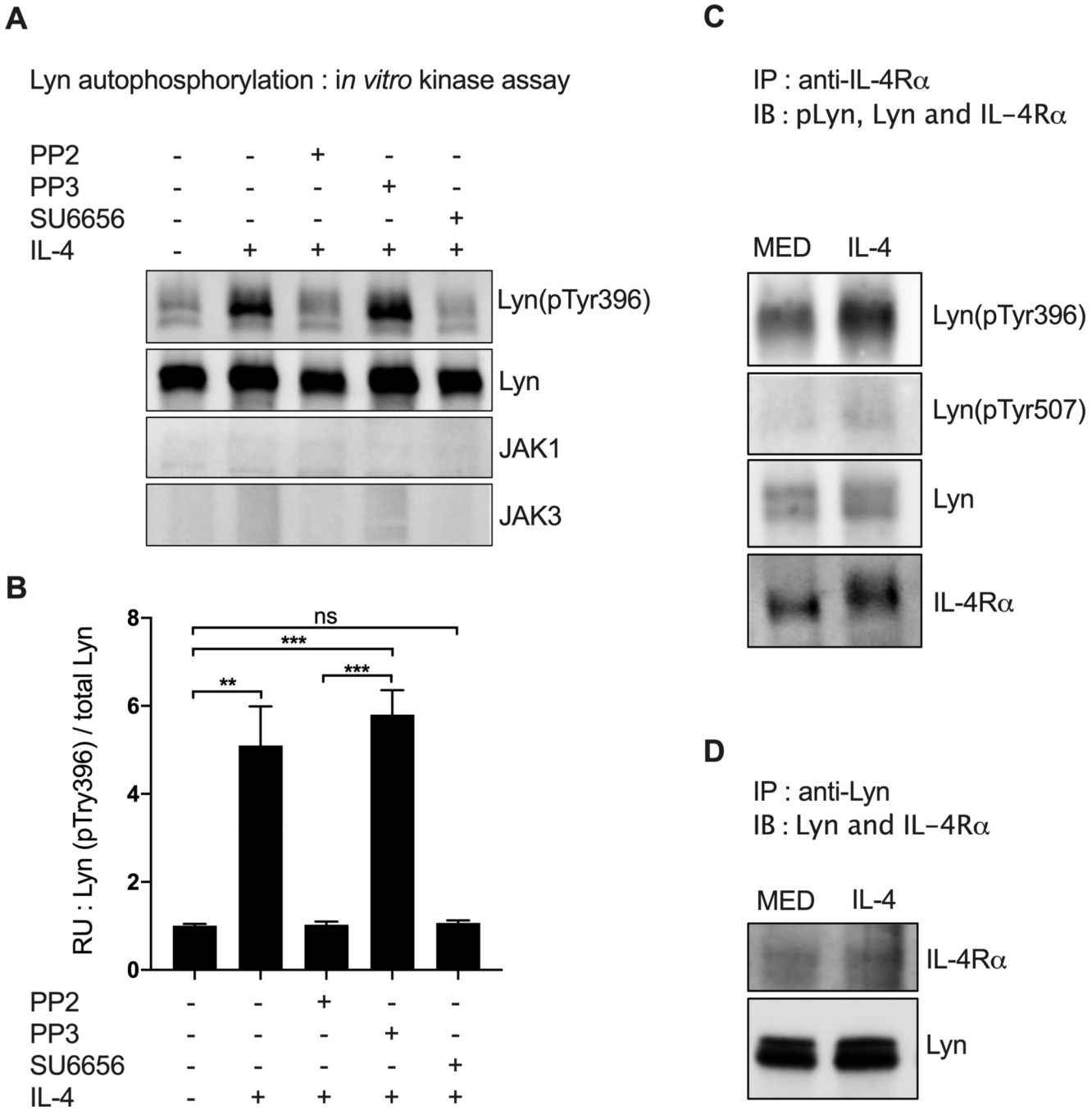

Figure 3. Lyn from IL-4 treated B cells autophosphorylates.

(**A**) Lyn autophosphorylation was evaluated by *in vitro* kinase assay at 30±C for 30 min by incubating immunoprecipitated Lyn from extracts of purified B cells cultured in medium for 3 h (–) or treated with IL-4 for 30 min in the presence of 20 μM PP2 or PP3 (negative control for PP2) and a selective Src family kinase inhibitor SU6656 (10 μM) for 1 h before IL-4 treatment. Immunoblotting with Lyn(pTyr396)-specific Ab was used to analyze Lyn autophosphorylation. Membranes were stripped and reprobed with Lyn-specific Ab as loading control. Further, the membrane was also stripped and reprobed with JAK1 and

JAK3-specific Abs to ensure that JAK kinases were not co-immunoprecipitated with Lyn. (**B**) Bar graphs show quantitation of Lyn(pTyr396) bands in relation to Lyn with the value of untreated B cells set at 1. (**C**) IL-4Rα was immunoprecipitated from extracts of purified B cells cultured in medium for 3 h (MED) or treated with IL-4 for 30 min. Immunoprecipitants were immunoblotted with Lyn(pTyr396), Lyn(pTyr507) and Lyn-specific Abs. (**D**) Lyn was immunoprecipitated from extracts of purified B cells cultured in medium for 3 h (MED) or treated with IL-4 for 30 min with anti-Lyn Ab. Immunoprecipitants were immunoblotted with IL-4Rα and Lyn-specific Abs. The experiment was performed at least three times with pooled B cells from three mice per group in each experiment, and data from a single representative experiment is shown. Values represent mean ± SD. Unpaired t test was performed to compare the data obtained for MED and IL-4 treated B cells. p values of < 0.05 were considered statistically significant. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

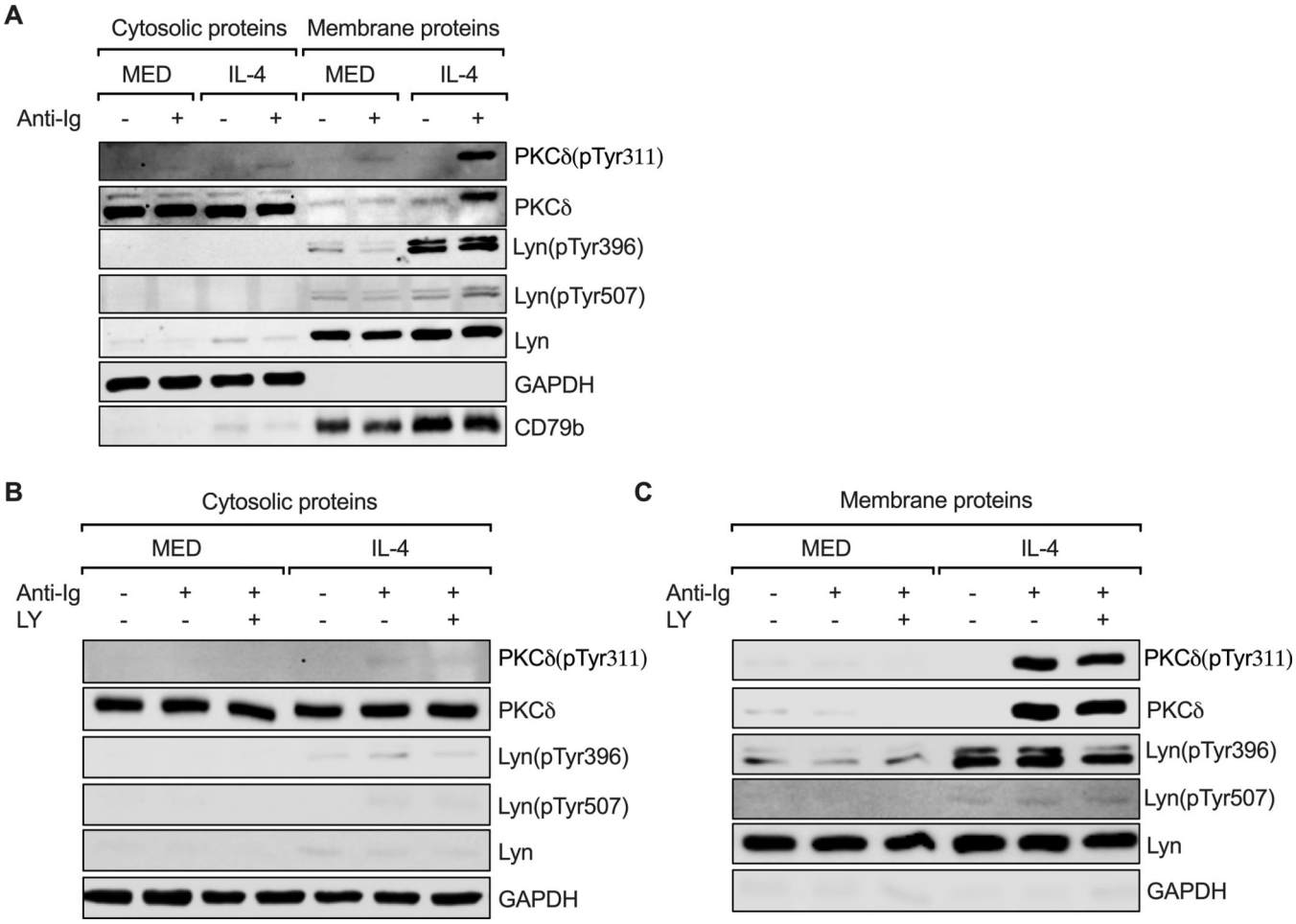

Figure 4: IL-4 and anti-Ig together induce membrane translocation and phosphorylation of PKCδ.

(**A**) Freshly purified B cells were cultured in medium for 3 h (MED) or treated with IL-4 for 24 h followed by anti-Ig stimulation for 0 (−) or 10 (+) min. Cytosolic and membrane protein extracts were prepared as described in Material and Methods. PKCδ protein expression and phosphorylation were measured by western blot analysis in cytosol and membrane extracts using Abs directed against PKCδ and PKCδ(pTyr311); respectively. Membranes were stripped and reprobed with Abs directed against Lyn, Lyn(pTyr396) and Lyn(pTyr507). GAPDH and CD79b (Igβ) specific Abs were used to measure the purity of the extracts and as loading controls. (**B** and **C**) Freshly purified B cells were cultured in medium for 3 h (MED) or treated with IL-4 for 24 h followed by anti-Ig stimulation for 0 (−) or 10 (+) min in the presence or absence of PI3K inhibitor (LY294002 (LY) (10 μM)) for 1 h before anti-Ig stimulation. Cytosolic (**B**) and membrane (**C**) protein extracts were prepared as described in Materials and Methods. PKCδ protein expression and phosphorylation were measured by western blot analysis in cytosol and membrane extracts using Abs directed against PKCδ and PKCδ(pTyr311); respectively. Membranes were stripped and reprobed with Abs directed against Lyn, Lyn(pTyr396), and Lyn(pTyr507). GAPDH-specific Ab was used to measure the purity of the extracts and as a loading control. The experiments were

performed at least three to four times with pooled B cells from three mice per group in each experiment, and data from a single representative experiment is shown.

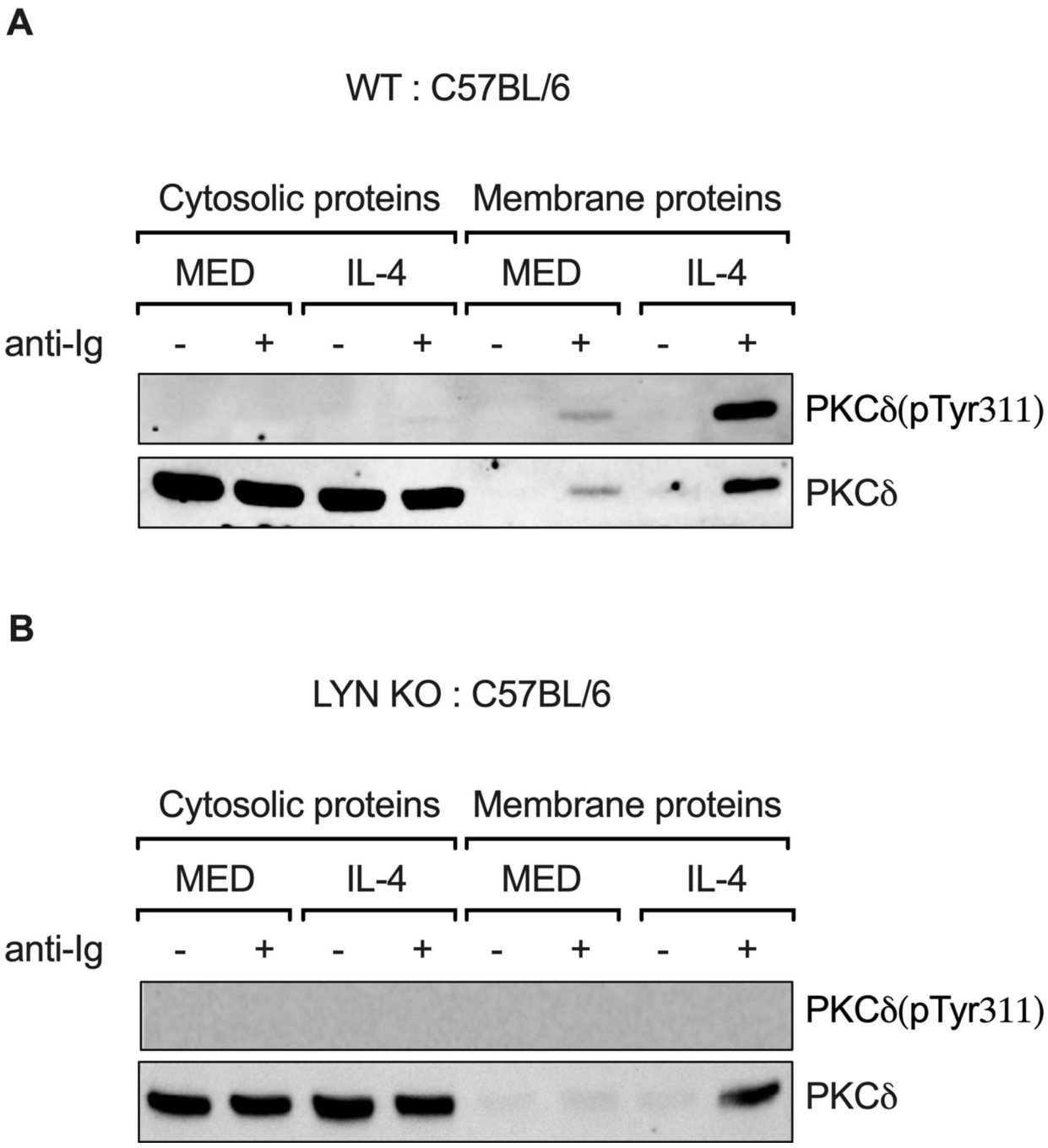

Figure 5: In the absence of Lyn, IL-4 and anti-Ig together induce membrane translocation but not phosphorylation of PKCδ.

Freshly purified B cells from WT (**A**) and Lyn KO (**B**) mice were cultured in medium for 3 h (MED) or treated with IL-4 for 24 h followed by anti-Ig stimulation for 0 (−) or 10 (+) min. Cytosolic and membrane protein extracts were prepared as described in Materials and Methods. PKCδ expression and phosphorylation were measured by immunoblotting using Abs directed against PKCδ and PKCδ(pTyr311); respectively. Experiments were performed

at least two to four times with pooled B cells from three to nine mice per group in each experiment, and data from a single representative experiment is shown.

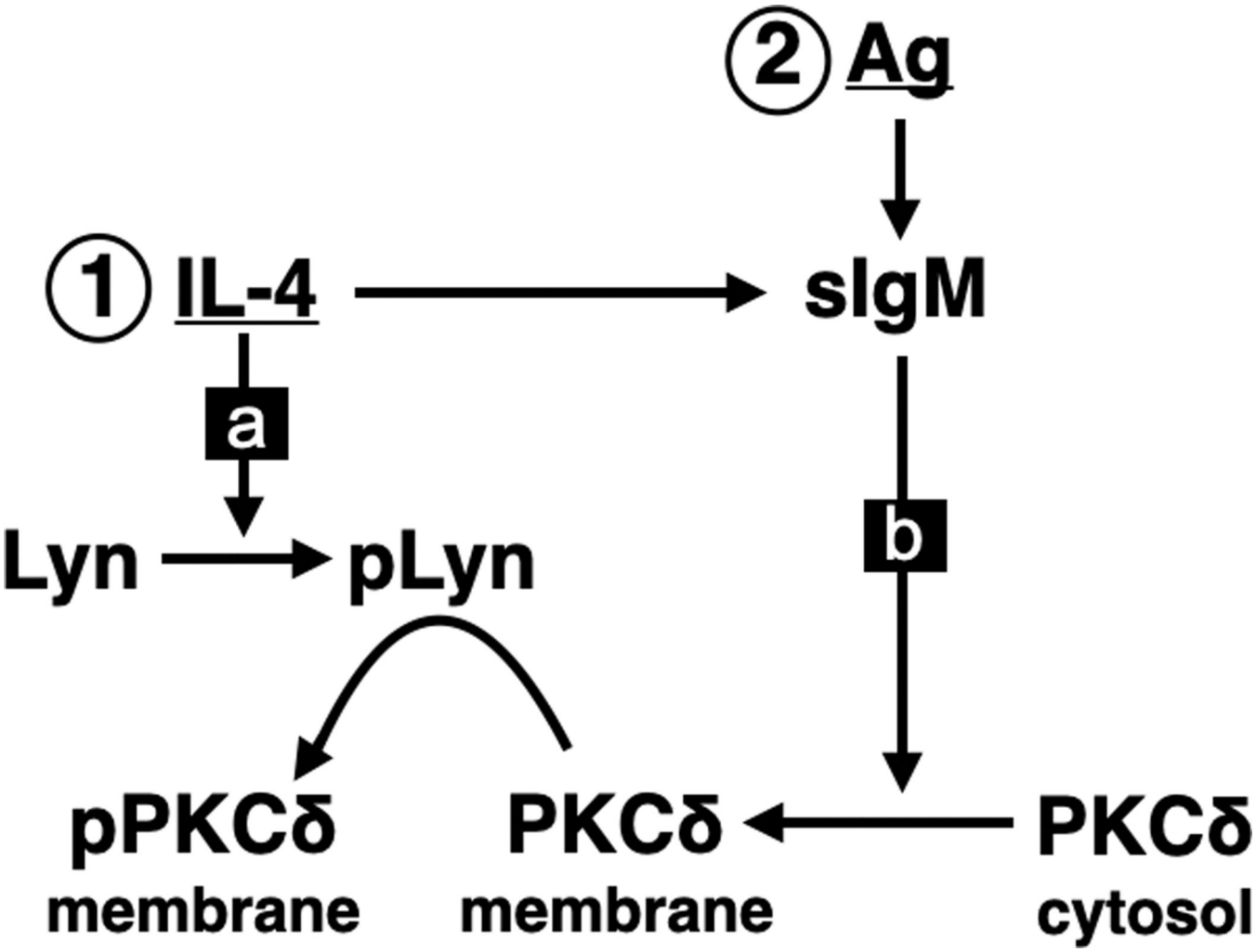

Figure 6. Signal integration by translocation and phosphorylation of PKCδ in the IL-4 induced alternate pathway for BCR signaling.

(1) The Lyn kinase directly associates with IL-4Rα in the B cell membrane. Following IL-4 treatment, Lyn becomes activated through autophosphorylation. (2) BCR signaling after B cell exposure to IL-4 produces translocation of PKCδ from the cytosol to the membrane. Once colocalized in the membrane, phosphorylated and activated Lyn phosphorylates PKCδ, similar to what happens when activated Lyn is forced together with PKCδ protein *in vitro*. Note that neither IL-4 nor anti-Ig working alone produce this outcome, but together achieve this result, so this process appears to model a molecular form of signal integration.