Involvement of SH2-SH2-SH3 domain of phospholipase Cγ1 in NF-κB signaling

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Abstract To directly define the role of phospholipase Cγ1 (PLCγ1) in NF-κB activation, NF-κB promoted luciferase reporter gene plasmid (pNF-κB-Luc) was transfected into rat-3Y1 fibroblasts that overexpress whole PLCγ1 (PLCγ1-3Y1), src homology domains SH2-SH2-SH3 of PLCγ1 (SH223-3Y1) and v-src (Src-3Y1). Transient transfection with pNF-κB-Luc remarkably increased the luciferase activity in all three transformants compared with normal rat-3Y1 cells. Pretreatment with inhibitors of protein tyrosine kinase reduced this increase in luciferase activity, but U73122 (a PLC inhibitor) did not. While PD98059, an inhibitor of mitogen activated protein kinase (MAPK), significantly reduced the luciferase activity, there was no effect by wortmannin and Ro-31-8220, inhibitors of phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC), respectively. This study shows a direct evidence that the SH2-SH2-SH3 region of PLCγ1 contributes to the NF-κB signaling and that MAPK, but not PI3K and PKC, is involved in SH2-SH2-SH3 mediated NF-κB activation in these cells.

Key words: Nuclear factor κB; Phospholipase Cγ1; Protein kinase C; Mitogen activated protein kinase; Phosphatidylinositol 3-kinase; SH2-SH3

1. Introduction

The REL/NF-κB/Iκ-B superfamily of signal transducers and transcription factors are paradigmatic of molecular mechanisms by which rapid responses in the immune system, cell proliferation and apoptosis, can be achieved [1–3]. A large number of stimuli, bacterial lipopolysaccharide, UV irradiation and reactive oxygen intermediates, can activate the NF-κB/REL signaling pathways [1,4,5]. In regulation of NF-κB signaling, in Jurkat T cells, the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI3K) has been shown to associate with the Tyr-42 residue on the Iκ-Bα, releasing NF-κB for its translocation [6]. Moreover, c-src was implicated in the tyrosine phosphorylation of Iκ-Bα in bone marrow macrophages [7]. Bacterial phospholipase C (PLC) was also reported to induce NF-κB activation [8].

Phospholipase Cγ1 (PLCγ1), one member of the PLC isozyme family, catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), creating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers stimulate the release of Ca^{2+} from an internal store and activate protein kinase C (PKC), respectively [9], ultimately leading to many biological processes. PLCγ1 contains two src homology domains 2 (SH2) and one src homology domain 3 (SH3), needed for effector activation or protein–protein interactions [10]. Rat-3Y1 cells overexpressing PLCγ1 (PLCγ1-3Y1) or SH2-SH2-SH3 domain of PLCγ1 (SH223-3Y1) exhibited morphological change to transformed cells and induced tumors when injected into nude mice [11]. Furthermore, treatment of CHO-K1 cells with lipopolysaccharide (LPS), DAG or bacterial PLC induced NF-κB activation [8] and there also were reports on the involvement of phosphatidylcholine specific PLC (PC-PLC) in NF-κB activation [12,13]. These results suggest the possible involvement of PLCγ1 in the activation of NF-κB signaling. However, there has been no direct evidence for the PLC involvement in the activation of NF-κB.

In this study, using the PLCγ1-3Y1, SH223-3Y1 and Src-3Y1 cells, we showed that SH2-SH2-SH3 domain of PLCγ1 is responsible for its effect on NF-κB signaling.

2. Materials and methods

2.1. Materials

Wortmannin, Ro-31-8220, PD98059, U73122 and tyrphostin A_{25} were purchased from Calbiochem, genistein and poly(dI-dC) were obtained from Sigma, Sephadex G-50, T4 polynucleotide kinase (T4PNK) and luciferase reporter gene assay kit (constant light signal) were from Boehringer Mannheim and lipofectamine was purchased from Gibco-BRL.

2.2. Plasmids and oligonucleotides

Plasmids pCRE-Luc, pNF-κB-Luc, pAP1-Luc, and pFC-MEKK were from Stratagene. These inducible vectors (5.7 kb) contain the luciferase reporter gene driven by a basic promoter element (TATA box) plus a defined inducible *cis*-enhancer element. Expression of the *Photinus pyralis* (firefly) luciferase gene in the reporter plasmid is controlled by a synthetic promoter that contains tandem repeats of cyclic AMP response element (CRE, 4×AGCC**TGACGTCA**GAG), and the binding sites for nuclear factor κB (NF-κB, 7×**TGACTAA**) and activator protein 1 (AP-1, 5×T**GGGGACTTTCC**GC). Constitutive expression of MEK kinase (MEKK) was driven by CMV promoter. Transcription consensus NF-κB, AP-1 and CREB oligonucleotides were obtained from Promega (Cat# E3291, E3201 and E3281, respectively).

2.3. Cell culture

PLCγ1-3Y1 and SH223-3Y1 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing an appropriate amount of hygromycin B supplemented with 10% of fetal bovine serum (FBS). Rat-3Y1 and Src-3Y1 cells were maintained in DMEM with 10% of FBS [11].

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2.4. Transfection of cells with pNF-κB-Luc, pAP1-Luc and pCRE-Luc

All procedures for the transfection were undertaken by slight modification of the liposome transfection method prescribed by the suppliers. Cells were seeded at a density of 1.5×10^3 cells/ml per well in DMEM supplemented with 10% fetal bovine serum in a 12-well plate and grown overnight in a humidified CO_2 incubator at 37°C. After washing with phosphate buffered saline (PBS), cells were loaded with 100 μl of liposomal DNA mixture and 400 μl of serum-free OPTI-MEM at 37°C for 24 h. For preparation of the liposomal DNA mixture, 2.5 μl of lipofectamine, 0.5 μg of reporter vectors and an appropriate amount of pFC-MEKK were mixed in 100 μl of serum-free OPTI-MEM for 45 min at room temperature. Meanwhile, test compounds (wortmannin, Ro-31-8220, U73122, tyrphostin A_{25} and genistein) were treated to the cells throughout the experiments for 24 h.

2.5. Determination of luciferase activity

After transfection for 24 h, cells were washed with PBS to remove the phenol red in the medium and refreshed with 100 μl of PBS. Cells were lysed by adding 100 μl of lysis solution of luciferase assay kit and the supernatants were counted by luminometer for luciferase activity expressed as relative light units (RLU).

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was done according to the procedure of Janssen and Sen [14]. Briefly, cells ($2–10\times10^6$) grown in complete medium were lysed on ice for 15 min in hypotonic buffer containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA, sodium salt), 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 0.3 mg/ml leupeptin, 1 mM dithiothreitol (DTT) and 0.6% Nonidet P-40. After centrifugation at $16\,000\times g$ for 1 min, the pellet of nuclei was resuspended in ice-cold high salt extraction buffer (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM PMSF, and DTT) for 30 min at 4°C. The nuclear lysates were then centrifuged at $16\,000\times g$ for 15 min at 4°C, and the supernatant was stored at −80°C or further proceeded for EMSA. NF-κB oligonucleotide (3.5 pmol) was incubated with 50 μCi [γ-^{32}P]ATP in the presence of 5 U T4 polynucleotide kinase and 1×kinase buffer supplied with the kinase (Boehringer) at 37°C for 30 min. The reaction was stopped by 50 mM EDTA, centrifuged through a Sephadex G-50 column to remove unincorporated ^{32}P, and stored at −20°C until use. For the DNA binding reaction and electrophoresis, nuclear protein extract (5 μg) was incubated in the presence of 0.03 pmol ^{32}P-endlabeled NF-κB oligonucleotide, 40 mM HEPES (pH 7.8), 10% (v/v) glycerol, 1 mM $MgCl_2$, 0.1 mM DTT, and 500 ng poly-(dI-dC) (Sigma) in a final volume of 10 μl for 20 min at room temperature. Following incubation, the sample was loaded on a 6% non-denaturing polyacrylamide gel in 0.5×Tris/boric acid/EDTA (TBE) buffer and gels were autoradiographed for band shift examination.

2.7. Measurement of total inositol phosphates (IPt)

Cells were fasted in inositol-free DMEM containing 1 μCi/ml of myo-[2-^{3}H]inositol for 24 h. Cells were rinsed twice and incubated in inositol-free DMEM containing 20 mM HEPES, pH 7.5, 20 mM LiCl and 1 mg/ml BSA at 37°C for 15 min. U73122 and wortmannin were treated to the cells for 15 min prior to stimulation with serum (10%) for further 30 min and the incubation was stopped by adding ice-cold 5% $HClO_4$. Inositol phosphates were extracted for 30 min on ice and the acid soluble fraction applied to a Bio-Rad AG 1-X8 anion exchange column. Inositol phosphates were eluted with varying concentrations of ammonium formate as previously described [15].

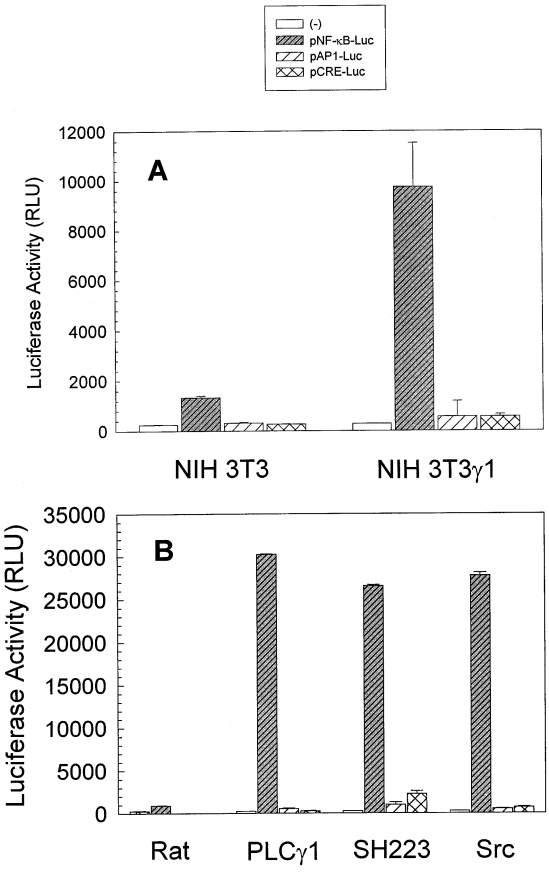

Fig. 1. Effect of PLCγ1, SH2-SH3 and v-src on NF-κB promoter activity. NIH 3T3 and NIH 3T3γ1 (A) or rat-3Y1, PLCγ1-3Y1 and SH223-3Y1 (B) cells, at a density of 1×10^3 cells/ml in a 12-well plate, were cultured for 24 h and thereafter transiently transfected with 1 μg of pNF-κB-Luc, pAP1-Luc and pCRE-Luc in OPTI-MEMI at 37°C for 24 h. The luciferase activity was measured using the constant light signal luciferase assay kit as described in Section 2. Each bar shows a representative from triplicate experiments.

3. Results

It was previously shown that overexpression of PLCγ1 in NIH 3T3 (NIH 3T3γ1) cells enhanced the platelet derived growth factor (PDGF) induced phosphoinositide hydrolysis

Table 1
Effect of inhibitors of PLC (U73122) and PTK (tyrphostin A_{25}) on inositol phosphate formation and NF-κB promoter directed luciferase activity in NIH 3T3 and rat-3Y1 cells

Inhibitor	Inositol phosphates (IPt)		Luciferase			
	NIH 3T3γ1	PLCγ1-3Y1	Rat-3Y1	PLCγ1-3Y1	SH223-3Y1	Src-3Y1
U73122	12 ± 2[a]	30 ± 5	85 ± 3	85 ± 5	85 ± 3	92 ± 4
Tyrphostin A_{25}	6 ± 1	21 ± 3	62 ± 4	41 ± 3	20 ± 3	35 ± 5

After semiconfluent growth, cells were labeled with 1 μCi/ml of myo-[2-^{3}H]inositol in inositol-free DMEM for 24 h, washed once with phosphate buffered saline and pretreated with U73122 (3 μM) or tyrphostin A_{25} (5 μM) for 15 min before serum stimulation for 30 min. The amount of intracellular inositol phosphate was measured as described in Section 2. On the other hand, cells were transfected with 1 μg of pNF-κB-Luc plasmid in OPTI-MEMI medium in the presence or absence of U73122 (3 μM) or tyrphostin A_{25} (5 μM) for 24 h and the luciferase activity was measured as in Fig. 1.

[a]All the values were expressed as a percentage to control values without the inhibitors and as means ± S.D. from a representative of triplicate experiments.

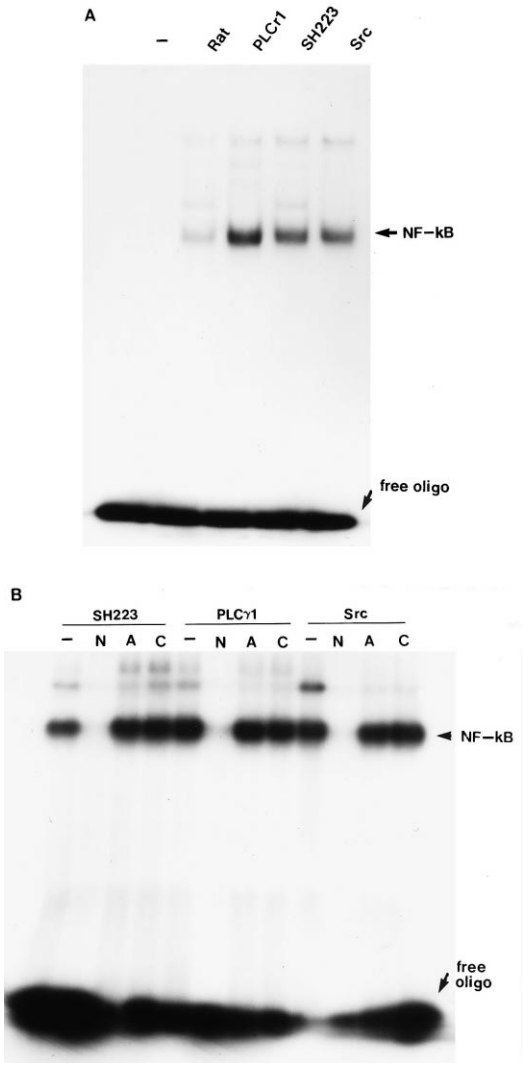

Fig. 2. Retardation of NF-κB oligonucleotide electrophoretic mobility by PLCγ1, SH2-SH2-SH3 domain, and v-src. A: Following lysis of confluent growth of cells, nuclear proteins (5 μg) were incubated with ^{32}P-labeled NF-κB consensus oligonucleotide (0.03 pmol, 100 000 μCi) for 20 min at room temperature and the reaction mixtures were applied to a 6% non-denaturing acrylamide gel. (−) indicates reaction without nuclear lysate. B: Reactions were performed in the absence (−) or presence of unlabeled consensus oligonucleotides NF-κB (N), AP-1 (A) and CRE (C) showing the specificity of NF-κB binding.

and phospholipase D (PLD) activities [16–18]. In addition, the SH2-SH3 domain of PLCγ1 as well as PLCγ1 itself caused tumorigenic growth of the transfected rat-3Y1 cells [11]. To see if PLCγ1 affect the NF-κB activation, NIH 3T3 and NIH 3T3γ1 cells were transiently transfected with pNF-κB-Luc containing the NF-κB promoter element and luciferase reporter gene (Fig. 1A). There was a remarkable increase in the amount of luciferase activity in NIH 3T3γ1 cells compared to NIH 3T3 cells. This was the same in rat-3Y1 fibroblasts (Fig. 1B). PLCγ1-3Y1, SH223-3Y1 and Src-3Y1 all showed greatly higher levels of luciferase activity than in rat-3Y1 cells when transfected with pNF-κB-Luc plasmid. However, the increase was not significant when transfected with pAP1-Luc and pCRE-Luc in all the transformed cells.

The results of luciferase reporter gene assay were consistent with those of gel mobility shift assay in Fig. 2. All three rat-3Y1 transformants showed a remarkable increase in NF-κB binding to its consensus oligonucleotide compared to normal rat-3Y1 cells (Fig. 2A). The binding specificity of NF-κB was confirmed by the observation that there was complete inhibition of NF-κB binding by 10-fold molar excess of unlabeled NF-κB but not by AP-1 and CRE consensus oligonucleotides in all the cells (Fig. 2B). To see if PLC is involved in the NF-κB activation process, U73122, an inhibitor of PLC [19,20], was treated to the rat cells together with the pNF-κB-Luc plasmid (Table 1). Unexpectedly, U73122 had only a negligible effect on luciferase activity while it reduced serum induced inositol phosphate formation by PLC in both NIH 3T3γ1 and PLCγ1-3Y1 cells. However, there was a significant reduction in luciferase activity by tyrphostin A_{25}, an inhibitor of protein tyrosine kinase [21] in all the cell types. This indicates that PLCγ1 enzyme activity itself is not crucial but SH2-SH3 domain of PLCγ1 is important for NF-κB activation possibly through the src homology mediated protein–protein interaction or protein tyrosine phosphorylations.

It was then necessary to determine what proteins or enzymes are involved in NF-κB signaling. PI3K was reported

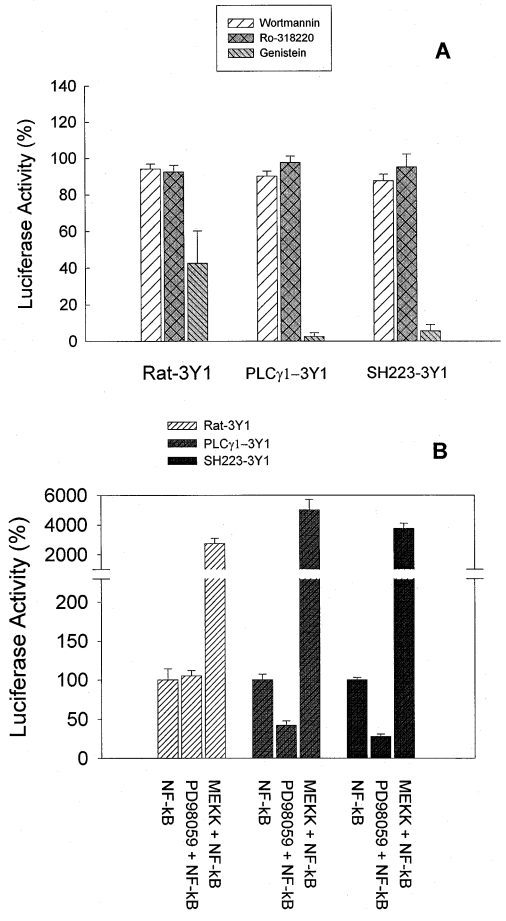

Fig. 3. Effect of inhibitors of PI3K, PKC, PTK and MAPK on NF-κB promoter activity. A: Just prior to pNF-κB-Luc transfection (1 μg), cells were treated with the inhibitors: wortmannin (2 μM), Ro-31-8220 (2 μM) and genistein (100 μM). B: Cells were cotransfected with pNF-κB-Luc (1 μg) and pFC-MEKK (50 ng) in the presence or absence of PD98059 (4 μM). All the other procedures were the same as in Fig. 1. In panel B, the control values at 100% for rat-3Y1, PLCγ1-3Y1 and SH223-3Y1 were 920 RLU, 30 000 RLU, and 26 000 RLU, respectively. Each bar shows a representative from triplicate experiments.

to associate with Iκ-Bα in a cell type specific manner [6,7] and PKC isoforms have also been implicated in NF-κB activation [12]. To examine the involvement of these enzymes in NF-κB signaling, wortmannin, Ro-31-8220 and genistein, inhibitors of PI3K, PKC and protein tyrosine kinase (PTK), respectively, were treated to the cells (Fig. 3A). It was observed that while genistein significantly reduced the luciferase activity, wortmannin (IC_{50}, 5 nM) and Ro-31-8220 (IC_{50}, 200 nM) had no effect even at concentrations of 2 μM.

It was reported that NF-κB can be activated by TRAF2 mediated MAP3K [22]. To test MAPK involvement in rat-3Y1 cells, cells were treated with PD98059, an inhibitor of MAPK pathway, or transfected with a MEKK containing plasmid, pFC-MEKK. As shown in Fig. 3B, MEKK increased NF-κB activation in all transfectants and the increase was much more pronounced in PLCγ1-3Y1 and SH223-3Y1 than in rat-3Y1 cells. On the contrary, as expected, PD98059 reduced the NF-κB mediated luciferase activity in all but rat-3Y1 cells.

4. Discussion

Although there have been reports for the agonist induced activation of PLCγ1 and NF-κB, direct evidence for the PLCγ1 mediated NF-κB activation has not been reported as yet. Some of the observations at best were with bacterial PLC or phosphatidylcholine specific PLC (PC-PLC) [8,12,13]. In this study, we used stable transfectants with mammalian phosphoinositide specific PLC (PI-PLC). NIH 3T3γ1 cells showed a higher level of phosphoinositide hydrolysis than NIH 3T3 cells in response to PDGF stimulation. However, without agonist, PLCγ1 was not activated in either the NIH 3T3 or NIH 3T3γ1 cells (data not shown) [16–18]. Regarding the NF-κB activation, however, NIH 3T3γ1 cells showed much higher level of NF-κB activity compared to NIH 3T3 cells even in serum-free state (Fig. 1A). This means that PLCγ1 activity is not relevant to NF-κB activation, which is further confirmed in Fig. 1B and Table 1 in which not only PLCγ1 itself but also the src homology domains of PLCγ1 greatly increased the NF-κB promoted luciferase activity. Furthermore, a PLC inhibitor U73122 did not affect the NF-κB activation. Thus, the SH2-SH2-SH3 domain in PLCγ1 appears to mediate the NF-κB activation. The contribution of the src homology domains to NF-κB activation was further supported in that v-src transformed Src-3Y1 cells also showed elevated level of luciferase activity and gel mobility shift (Figs. 1B and 2).

In regards to PI3K and PKC, wortmannin and Ro-31-8220 were treated to the rat cells. PI3K was known to bind to tyrosine phosphorylated Iκ-Bα and wortmannin inhibited this interaction in pervanadate stimulated Jurkat T cells [6]. However, we did not notice any inhibition of NF-κB promoted luciferase activity even at high concentrations of wortmannin. PKC-ζ was shown to phosphorylate Iκ-Bα in vitro [23], and in vivo studies with constitutively active isoforms demonstrated novel PKC-ε to be a potent inducer of a NF-κB dependent reporter gene [24]. In this study, however, treatment of a PKC inhibitor, Ro-31-8220, did not reduce NF-κB activity (Fig. 3A), and we also had the same result with calphostin C (data not shown). Thus, it is possible that active forms of PKC isozymes in quiescent rat-3Y1 cells could be PKC-ζ and λ since atypical PKC-ζ and λ are independent of Ca^{2+} and DAG for their enzyme activities and do not induce Iκ-Bα phosphorylation [25]. Another possibility could be raised but these assumptions should be exploited in a further study.

MAPK is another Ser/Thr kinase involved in NF-κB activation, although bifurcation of NF-κB and c-Jun N-terminal kinase (JNK/SAPK) pathways at TRAF2 was reported previously [22]. From the results in Fig. 3B, it appears that the SH2-SH2-SH3 domain of PLCγ1 makes the cells more susceptible to MAPK activity. This can be supported by the observation that an MEK inhibitor PD98059 reduced NF-κB activity in both PLCγ1-3Y1 and SH223-3Y1 cells but not in rat-3Y1 cells. Hence, MAPK seems to be directly or indirectly associated with PLCγ1.

Recently, it was reported that an SH3 containing rac target POSH leads to the nuclear translocation of NF-κB [26]. Besides, Fyn was found to activate NF-κB signaling through its intact SH2 domain [27], implying the involvement of src homology domains in NF-κB signaling pathway. In this study, however, we clearly demonstrate that PLCγ1 can mediate the NF-κB activation but the SH2-SH2-SH3 domain of the enzyme is responsible for NF-κB activation, and that PTK(s) and MAPK but not PI3K and PKC are involved directly or indirectly in this process.

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