Requirement of Phospholipase C-γ2 Activation in Surface Immunoglobulin M–induced B Cell Apoptosis

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Summary

Surface IgM (sIgM) stimulation induces the tyrosine phosphorylation of multiple cellular substrates, including phospholipase C (PLC)–γ2, which is involved in the activation of phosphatidylinositol pathway. DT40 B cells underwent apoptotic cell death when activated through sIgM, a phenomenon that is related to elimination of self-reactive B cells. To examine the roles of PLC-γ2 in sIgM signaling, we have generated DT40 cells deficient in PLC-γ2 Cross-linking of sIgM on PLC-γ2–deficient cells evoked neither inositol 1,4,5-trisphosphate nor calcium mobilization. In PLC-γ2– or Syk-deficient DT40 cells, the induction of apoptosis was blocked, but was still observed in Lyn-deficient cells. Src homology 2 domains of PLC-γ2 were essential for both its activation and sIgM-induced apoptosis. Since tyrosine phosphorylation of PLC-γ2 is mediated by Syk, these results indicate that activation of PLC-γ2 through Syk is required for sIgM-induced apoptosis.

Stimulation of surface IgM (sIgM)[1] on B cells by antigen or anti-IgM antibody initiates a cascade of biochemical events including protein tyrosine kinase (PTK) activation, phosphatidylinositol (PtdIns) hydrolysis, and calcium mobilization (for review see references 1–6). Functional analysis of the intracytoplasmic domains of Igα and Igβ, which are subunits of the sIgM complex, revealed that an 18–amino acid motif based on a tandem YXXL stretch, the immunoreceptor tyrosine-based activation motif (ITAM), couples the sIgM complex to these early signaling events (7–11). Two types of PTKs, Src-PTK and Syk kinase, are shown to associate with the sIgM complex through Igα/Igβ heterodimer (12–22). It has also been demonstrated that both Src-PTK and Syk are required for coupling sIgM to the induction of protein tyrosine phosphorylation and that these enzymes mediate the phosphorylation of at least partially distinct sets of substrates (23). One of the phosphorylated substrates mediated by Syk is phospholipase C (PLC)-γ2 (23–28). Phosphorylation of PLC-γ2 is responsible for an increase in its catalytic activity (29, 30). Activated PLC-γ2 catalyzes the hydrolysis of phosphoinositides, leading to the generation of second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (31, 32).

Calcium mobilization induced by sIgM ligation is a biphasic response composed of an initial release of internally stored calcium, and a subsequent entry of extracellular calcium (31, 33). Although it is widely accepted that $InsP_3$ is involved in release from intracellular calcium stores through binding to its receptor, it has not been well established that the calcium influx is also a downstream event of PLC-γ2 activation (34).

To avoid production of autoantibodies, two key censoring mechanisms are known to operate in B lymphocyte repertoires: functional silencing of self-reactive cells, termed clonal anergy, and physical elimination of self-reactive cells, termed clonal deletion (for review see references 35–37). The existence of such an irreversible censoring mechanism (clonal deletion) was first suggested by the failure of a mature B cell repertoire to develop in chickens and mice treated from hatching or birth with antibodies to IgM (38).

Elimination of B cells bearing self-reactive Ig molecules is initiated by the binding of self antigens to sIgM on immature B cells. Indeed, cross-linking of sIgM on immature B cell lines such as WEHI-231 induces apoptosis (39–47). However, it has not been elucidated which of the early biochemical signals are critical for inducing apoptosis upon sIgM cross-linking.

We have previously established Lyn- and Syk-deficient cells from chicken DT40 B cell line by gene targeting, exploiting unusually high homologous recombination proficiency of this cell line (23, 48). To further dissect the pathways downstream of PTK activation in sIgM signaling, we established a mutant B cell line that lacks PLC-γ2. Upon sIgM cross-linking, PLC-γ2–deficient cells completely

[1] *Abbreviations used in this paper:* $InsP_3$, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PLC, phospholipase C; PTK, protein tyrosine kinase; SH2, Src homology 2; sIgM, surface immunoglobulin M.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/10/907/08 $2.00
Volume 182 October 1995 907–914

abolished calcium mobilization and PtdIns hydrolysis. By transfection of the PLC-γ2 cDNA with Src homology 2 (SH2) mutations, we show that the SH2–phosphotyrosine interaction is essential for PLC-γ2 activation in sIgM signaling. Moreover, our results clearly indicate the requirement of PLC-γ2 activation for sIgM-induced apoptosis.

Materials and Methods

Cells, Expression Constructs, and Antibodies. Wild-type and various mutant DT40 cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, and glutamine. Construction of chicken expression vector pApuro was already described (23). Mutations in SH2 domains of rat PLC-γ2 cDNA were created by PCR. The resulting constructs were confirmed by DNA sequencing. The mutant and wild-type PLC-γ2 (24) and M1 muscarinic receptor (49) cDNAs were subcloned into pApuro vector and were electroporated into PLC-γ2–deficient DT40 cells. Selection was performed in the presence of puromycin (0.5 μg/ml). Expression of transfected cDNAs was confirmed by Western blot analysis (PLC-γ2) or binding assay (M1 muscarinic receptor). Anti–chicken IgM mAb M4 (50) and anti–rat PLC-γ2 antisera (51) were already described. Antiphosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Generation of PLC-γ2–deficient DT40 Cells. Chicken PLC-γ2 and PLC-γ2 cDNA clones were obtained by screening a chicken spleen cDNA library (Clontech Laboratories Inc., Palo Alto, CA) with rat PLC-γ2 cDNA under a low stringent condition. Cloned genomic DNA corresponding to the chicken PLC-γ2 locus was isolated from a library of chicken liver DNA (Clontech Laboratories Inc., Palo Alto, CA). The targeting vectors, pPLC-neo and pPLC-hisD, were constructed by replacing the 4-kb genomic sequence, which contains exons corresponding to amino acid residues 857–947 of rat PLC-γ2 cDNA, with *neo* or *hisD* cassette, respectively. The upstream 1.3 kb genomic sequence was generated by PCR using oligonucleotides containing XbaI restriction sites and was subcloned as an XbaI-XbaI fragment. The upstream sequence was a 6.2-kb BamHI-BglII genomic fragment. pPLC-neo was linearized and transfected into DT40 cells by electroporation (550 V, 25 μF). G418 selection (2 mg/ml) was started 24 h after transfection. Cells were cultured for ~14 d, expanded, and screened by Southern blot analysis. Similarly, pPLC-hisD was transfected into neo-targeted clone, and selection was performed with both G418 (2 mg/ml) and histidinol (1 mg/ml).

Western and Northern Blot Analyses. Cells were solubilized in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10 μM molybdate, 0.2 mM sodium vanadate supplemented with protease inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 0.5 mM benzamidine hydrochloride, 10 μg/ml chymostatin, 0.1 mM N-α-*p*-tosyl-L-lysine-chloromethyl ketone, 0.1 mM *N*-1-tosylamide-2-phenylethylchloromethyl ketone, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml calpain inhibitor I, and 10 μg/ml pepstatin). PLC-γ2 protein was immunoprecipitated by sequential incubation with anti–PLC-γ2 serum and protein A–Sepharose. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies. Filters were developed with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). RNA was prepared from wild-type and mutant DT40 cells using guanidium thiocyanate method. Total RNA (20 μg) was separated by 1.2% agarose, transferred to a Hybond-N membrane, and probed with a chicken PLC-γ2 cDNA fragment.

In Vitro PLC Assay. PLC activity was assayed by quantitating $InsP_3$ production (27). Briefly, PLC-γ2 protein was immunoprecipitated from the transfected COS cell lysate using specific antisera as described above, washed extensively, resuspended in PLC assay buffer (20 mM Na_2PO_4, pH 6.8, 70 mM KCl, 0.125% octylglucoside, 0.8 mM EGTA, 0.8 mM $CaCl_2$). Substrate containing 5 Ci/mmol [^{3}H]phosphatidylinositol 4,5-bisphosphate (Amersham) was added, then reaction was allowed to proceed at 37°C for 30 min and was stopped by adding TCA and BSA. After centrifugation, radioactivity in the supernatant was assayed by a liquid scintillation counter.

Calcium Measurements. Cells (5×10^6) were loaded with 3 μM fura-2/AM in PBS containing 20 mM Hepes, pH 7.2, 5 mM glucose, 0.025% BSA, and 1 mM $CaCl_2$ at 37°C for 45 min. Cells were then washed twice and adjusted to 10^6 cells per ml. Fluorescence of the cell suspension was monitored continuously with a fluorescence spectrophotometer (F-2000; Hitachi Instruments, Inc., San Jose, CA) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calcium levels were calibrated and calculated as described (52).

Phosphoinositides Analysis. Cells (10^6/ml) were labeled with myo-[^{3}H]inositol (10 μCi/ml, 105 Ci/mmol; Amersham) for 6 h in inositol-free RPMI 1640 supplemented with 10% dialyzed FCS, then stimulated with M4 mAb in the presence of 10 mM LiCl. The soluble inositol phosphates were extracted with TCA at indicated time points and were applied to AG 1-X8 (formate form) ion exchange columns (Bio Rad Laboratories, Richmond, CA) pre-equilibrated with 0.1 M formic acid. The columns were washed with 10 ml water and 10 ml of 60 mM ammonium formate/5 mM sodium tetraborate. Elution was performed with increasing concentrations of ammonium formate (0.1–0.7 M) (53).

DNA Fragmentation Assay. After treatment of cells with or without M4 mAb, cells (~1×10^6) were pelleted in 1.5-ml tubes. Pellets were gently resuspended in 20 μl of 10 mM EDTA/50 mM Tris-HCl, pH 8.0, containing 0.5% sodium laurylsarkosinate and 0.5 mg/ml proteinase K, and incubated for 2 h at 50°C. RNaseA (10 μl, 0.5 mg/ml) was added to each sample and incubation was continued for an additional 2 h at 50°C. Samples were heated to 70°C and mixed with 10 μl of 10 mM EDTA containing 1% low melting–temperature agarose, 0.25% bromophenol blue, and 40% sucrose before applying into 2% agarose gel (54).

Flow Cytometric Analysis. For DNA content analysis, stimulated or unstimulated cells (~1×10^6) were pelleted, and resuspended in 1.5 ml hypotonic DNA staining solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Samples were kept at 4°C overnight, and subjected to analysis by FACSort® (Becton Dickinson & Co., Mountain View, CA). Debris and doublets were excluded by appropriate gating (55). For cell surface expression of sIgM, DT40 cells were washed, subsequently incubated with FITC-conjugated anti–chicken IgM (Bethyl Laboratories, Inc., Montgomery, TX), and analyzed using FACSort.

Binding Assay for M1 Muscarinic Receptor Expression. Cells (~10^6) were incubated for 90 min with muscarinic receptor agonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB, 100 pM, 47 Ci/mmol; Amersham) at room temperature. After incubation, cells were collected on a GF/B membrane (Whatman Inc., Clifton, NJ) and washed extensively. Bound radioactivity was determined by a liquid scintillation counter. All samples were assayed in duplicate,

and background binding activity was determined in the presence of 10 μM atropine.

Results

Targeted Disruption of PLC-γ2. To examine the roles of PLC-γ2 in sIgM signaling, we established DT40 cells deficient in PLC-γ2 by gene targeting. For disruption of the PLC-γ2 gene locus, the mutations of the two PLC-γ2 alleles were introduced in DT40 cells by sequential homologous recombinations (Fig. 1, *A–C*). The targeting vectors have a neomycin or histidinol resistance gene cassette replacing the chicken genomic sequence, which contains exons corresponding to the region that is essential for PLC-γ2 catalytic activity (Fig. 1 *B*) (24). Homologous recombination events were screened by Southern blot analysis, and two independent clones bearing homozygous mutations at the PLC-γ2 locus were identified (Fig. 1 *D*). Southern blot analysis revealed a single integration of each vector in these clones (data not shown). To verify null mutations, Northern and Western blot analyses using specific probes were carried out. Both RNA and protein expression of PLC-γ2 were abrogated in these mutant cell clones (Fig. 1, *E* and *F*). Furthermore, Northern blot analysis showed that PLC-γ2 is not expressed in DT40 cells (data not shown). The level of cell surface expression of sIgM on the PLC-γ2–targeted clone was essentially the same as that of parental DT40 cells (Fig. 2).

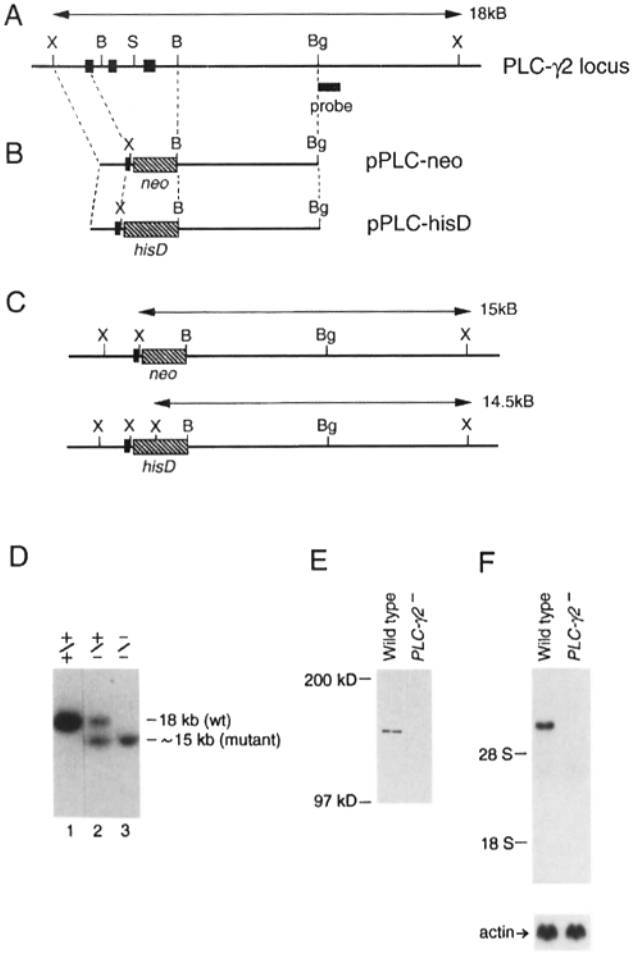


Figure 1. Gene targeting of the chicken PLC-γ2 locus. (*A*) Partial restriction map of PLC-γ2 locus. (*B*) Structure of the targeting vectors pPLC-γ2 neo and pPLC-hisD. (*C*) Predicted structure of the targeted PLC-γ2 locus. Only the exons that are disrupted or deleted are indicated as black boxes. The location of the hybridization probe and expected sizes of the XbaI fragments that hybridize with the probe are shown. *X*, XbaI; *B*, BamHI; *Bg*, BglII; *S*, SacI. (*D*) Southern blot analysis of wild-type DT40 cells (+/+, lane *1*), neo-targeted cells (+/−, lane *2*), *neo*- and *hisD*-targeted cells (−/−, lane 3). Genomic DNA was digested by XbaI. (*E* and *F*) Expression of PLC-γ2 in wild-type and targeted DT40 cells. (*E*) PLC-γ2 protein was immunoprecipitated with anti–PLC-γ2 serum and was detected by Western blot analysis. Positions of molecular weight standards are shown. (*F*) RNA expression was analyzed by Northern blot analysis using chicken cDNA probes for PLC-γ2 (*upper panel*) or β-actin (*lower panel*) (56). Positions of 28S and 18S RNA are shown.

PLC-γ2 Activation Is Essential for both Ca^{2+} Release from Intracellular Pools and Ca^{2+} Influx. To assess the contribution of PLC-γ2 to sIgM-induced signaling, we first analyzed early events in PLC-γ2–deficient DT40 cells. Cells were stimulated with anti–chicken IgM mAb (M4), and induction of protein tyrosine phosphorylation was analyzed by immunoblotting with antiphosphotyrosine mAb 4G10. Compared with wild-type DT40, PLC-γ2–deficient cells showed an essentially similar pattern of tyrosine phosphorylation (Fig. 3 *A*), indicating that PLC-γ2 does not affect the global tyrosine phosphorylation upon receptor cross-linking. In contrast to wild-type cells, PLC-γ2–deficient cells abrogated PtdIns hydrolysis upon receptor stimulation (Fig. 4 *B*), providing the direct evidence that PLC-γ2 is responsible for sIgM-induced PtdIns pathway. In addition, M4-induced calcium mobilization was completely abolished (Fig. 4 *A*). Loss of PLC-γ2 did not affect calcium

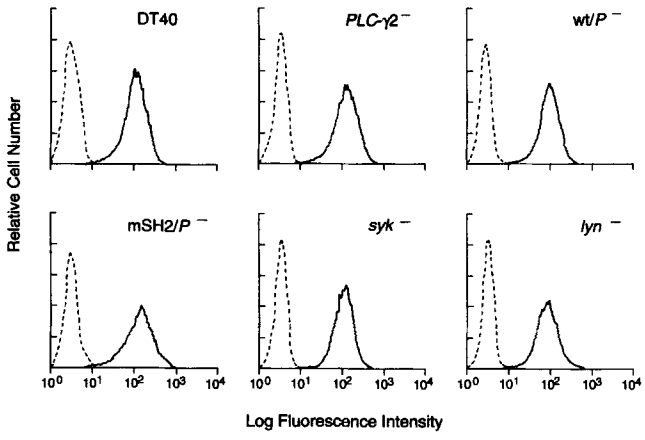


Figure 2. Cell surface expression of sIgM on various DT40 mutant cells. DT40 cells were stained with FITC-conjugated anti–chicken IgM. Unstained cells were used as negative controls. PLC-γ2–deficient cells expressing wild-type and SH2 mutant of PLC-γ2 are indicated as wt/P^- and mSH2/P^-, respectively.

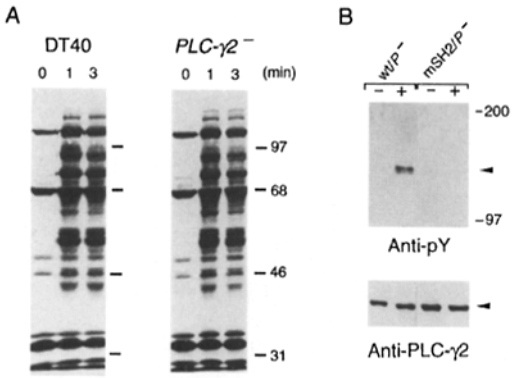
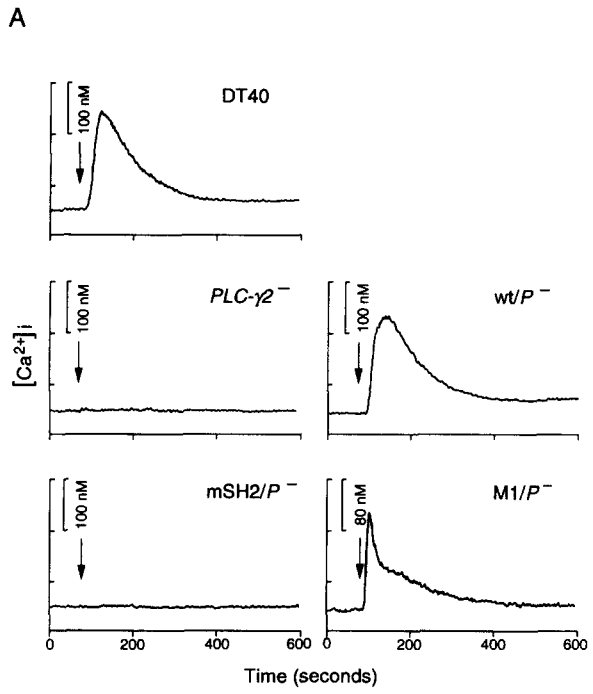


Figure 3. Tyrosine phosphorylation of whole-cell lysate and PLC-γ2 in various DT40 cells after activation with anti-IgM. (*A*) At the indicated times after the addition of M4 (4 μg/ml), whole-cell lysates prepared from 2.5 × 10^6 cells were loaded onto an 8% SDS-PAGE gel. (*B*) PLC-γ2 was immunoprecipitated from cells (2 × 10^6 cells per lane) before stimulation (−) or after stimulation (+) with M4 (4 μg/ml) for 3 min, and the immunoprecipitates were separated on a 6% SDS-PAGE gel. After transfer to nitrocellulose, the filters were incubated with antiphosphotyrosine mAb 4G10. To check the content of PLC-γ2 (*B, lower panel*), the filter was reprobed with anti–PLC-γ2 Ab after deprobing.

mobilization induced by stimulation of M1 muscarinic receptor (49), as shown by transfectant with M1 receptor in PLC-γ2–deficient cells (Fig. 4 *A*). These observations demonstrate that both calcium release from intracellular pools and entry across the plasma membrane are the downstream events of PLC-γ2 activation in sIgM signaling.

SH2 Domains of PLC-γ2 Are Required for its Activation through sIgM Stimulation. Tyrosine kinase growth factor receptors interact with PLC-γ2 by recruiting the enzyme via its SH2 domains to the tyrosine-phosphorylated receptor (57, 58). To examine the role of SH2 domains of PLC-γ2 in signaling responses upon sIgM stimulation, we mutated the phosphotyrosine-binding site in the two SH2 domains of rat PLC-γ2 (both Arg^{564} and Arg^{672} to Ala; mSH2). This mutated cDNA was transfected into PLC-γ2–deficient DT40 cells (mSH2/P^+). As shown in Fig. 3 *B*, expression extent of mutated PLC-γ2 was about the same level as that of the transfectant with wild-type PLC-γ2 (wt/P^+). In the transfectant with wild-type PLC-γ2, sIgM-induced calcium mobilization was restored, and the PtdIns hydrolysis was more vigorous than that in wild-type DT40 cells (Fig. 4, *A* and *B*). This is probably caused by the overexpression of PLC-γ2 since this hydrolysis in another transfectant expressing lower level of wild-type PLC-γ2 was less remarkable (data not shown). In contrast to wild-type PLC-γ2, the SH2 mutant of PLC-γ2 was unable to evoke both PtdIns hydrolysis and calcium mobilization upon sIgM stimulation (Fig. 4, *A* and *B*). The inability of this mutant to induce PtdIns hydrolysis correlated well with the mutated PLC-γ2 molecule's loss of sIgM-induced tyrosine phosphorylation (Fig. 3 *B*). Catalytic activity of the SH2 mutant of PLC-γ2 was comparable to that of wild-type PLC-γ2 in COS cells (data not shown). These observations suggest that recruitment of PLC-γ2 to phosphorylated tyrosine via its SH2 domains is a prerequisite for sIgM-induced tyrosine phosphorylation of PLC-γ2.

Activation of PLC-γ2 through Syk Is Required for sIgM-coupled Apoptosis. It is well known that sIgM stimulation eventually leads to growth inhibition and apoptosis in immature B cell lines. Thus, we sought to examine whether sIgM stimulation can induce apoptosis in this cell line. Treatment of wild-type DT40 cells for 24 h with mAb M4 resulted in a drastic increase of the percentage of apoptotic



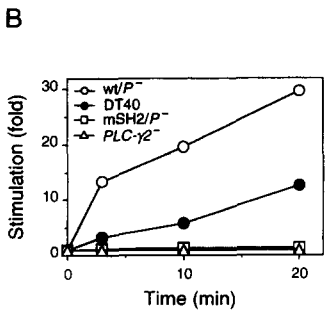


Figure 4. Early signaling events in wild-type and mutant DT40 cells. (*A*) Intracellular free calcium mobilization. fura-2–loaded cells were monitored by a spectrophotometer upon stimulation with M4 (2 μg/ml) or carbachol (500 μM). Arrows indicate time points at which stimulants were added. PLC-γ2–deficient cells expressing M1 muscarinic receptor are indicated as M1/P^-. (*B*) $InsP_3$ generation. Soluble inositol were extracted from cells stimulated with M4 and were separated by AG1-X8 anion exchange columns. Data are shown as fold increase of the value before stimulation with M4.

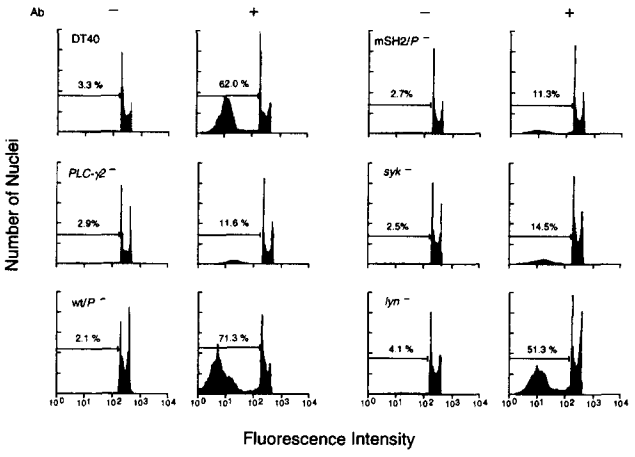


Figure 5. Induction of apoptosis in wild-type and mutant DT40 cells. Cells were cultured with (+) or without (−) M4 (10 μg/ml, 24 h), treated in hypotonic DNA staining solution containing 50 μg/ml propidium iodide and were subjected to analysis by FACSort® (Becton- Dickinson). The percentage of fragmented nuclei is indicated.

cells, as assessed by propidium iodide staining and flow cytometric analysis (Fig. 5) (55). Also, analysis of genomic DNA from anti-IgM–treated DT40 cells revealed a characteristic fragmentation pattern of nucleosomal ladder on agarose gel (data not shown). These results indicate that DT40 cells undergo apoptosis by signaling through sIgM.

PLC-γ2–deficient DT40 cells, along with Syk- or Lyn-deficient cells, were treated with mAb M4 for 24 h and the induction of apoptosis was analyzed. Stimulation of Lyn-deficient cells with M4 induced apoptotic cell death similar to wild-type cells, whereas Syk- or PLC-γ2–deficient cells treated with M4 showed a dramatically reduced percentage of fragmented nuclei (Fig. 5). This blockade of apoptosis was not caused by low level expression of sIgM in Syk- or PLC-γ2–deficient cells because both cells expressed the same level of sIgM as wild-type cells (Fig. 2). Furthermore, transfection of Syk or PLC-γ2 cDNA into these deficient cells restored the defect (Fig. 5 and data not shown).

Since the SH2 domains of PLC-γ2 are essential for Syk-mediated PLC-γ2 activation in sIgM signaling, we examined the effect of the SH2 mutation on sIgM-induced apoptosis. Similar to PLC-γ2–deficient cells, cells expressing the SH2 mutant of PLC-γ2 showed the blockade of sIgM-induced apoptosis (mSH2/P$^+$ in Fig. 5). These observations demonstrate that activation of PLC-γ2 through Syk is critical for sIgM-induced apoptosis.

Discussion

Lyn- or Syk-deficient DT40 cells respond to sIgM stimulation with a limited tyrosine phosphorylation of cellular substrates (23), whereas PLC-γ2–deficient cells exhibited almost the same pattern of tyrosine phosphorylation as the wild- type cells upon sIgM cross-linking (Fig. 3 *A*). This observation, together with the evidence that sIgM-induced tyrosine phosphorylation of PLC-γ2 is dependent on Syk (23), supports the concept that PLC-γ2 activation is a downstream event of PTK activation in sIgM signaling.

Experiments in which PLC-γ2 cDNA with SH2 mutations was introduced into PLC-γ2–deficient cells demonstrate that the SH2 domains are critical for activation of PLC-γ2 through sIgM. Since sIgM-induced phosphorylation of PLC-γ2 was also abolished in this SH2 mutant (Fig. 3 *B*), it appears that SH2-phosphotyrosine interaction is a prerequisite for phosphorylation of PLC-γ2. These observations raise the possibility that, as in the case of receptor tyrosine kinase signaling (57, 58), PLC-γ2 is recruited via its SH2 domains to inducibly phosphorylated proteins such as sIgM complex or associated PTKs. Indeed, various phosphoproteins, including Src-PTKs, are shown to associate with SH2 domains of PLC-γ2 isoform in vitro (59–64). Because tyrosine phosphorylation of PLC-γ2 is still induced by sIgM stimulation in Lyn-deficient DT40 cells (23), candidates to which PLC-γ2 is recruited include those inducibly phosphorylated proteins in Lyn-deficient cells.

Rapid calcium mobilization following sIgM stimulation presents two phases: the initial phase consists of a transient release of calcium from intracellular stores and is followed by a sustained calcium influx caused by the opening of the calcium channels present in the plasma membrane (31, 33). Although it has been well established that $InsP_3$ is primarily responsible for mobilizing calcium from intracellular storage sites through binding to its receptor, the regulatory mechanisms for calcium entry are less clear (34). The prevailing hypothesis is that calcium entry is somehow coupled to the $InsP_3$-induced depletion of intracellular calcium stores, a process termed capacitative calcium entry. The most compelling evidence for this hypothesis comes from the observation that inducers of intracellular calcium pool depletion, such as thapsigargin, mimic the ability of surface receptors to activate calcium entry (33). On the other hand, receptor-operated calcium mobilization in the absence of detectable PtdIns hydrolysis have previously been noted in several systems, suggesting the presence of $InsP_3$-independent calcium entry mechanism (65–69). Abolishment of sIgM-induced calcium mobilization in PLC-γ2–deficient cells (Fig. 4 *A*) provides the direct evidence that both calcium release from endoplasmic reticulum and entry from outside of the cells are downstream events of PLC-γ2 activation. This conclusion supports the capacitative calcium entry hypothesis, and the previously observed discrepancy might be explained by relative insensitivity of PtdIns assays.

In this study, we have demonstrated that activation of PLC-γ2 is required for sIgM-induced apoptosis. The blockade of sIgM-induced apoptosis in Syk-deficient DT40 cells supports this conclusion because Syk, not Lyn, is essential for coupling sIgM to the PtdIns pathway (23). The requirement of PLC-γ2 activation suggests that second messengers, $InsP_3$ and diacylglycerol, as well as calcium mobilization, are crucial for the induction of apoptosis. In-

deed, sustained calcium elevation has been implicated in apoptosis of various cell systems (70, 71). It appears unlikely, however, that these second messengers alone initiate apoptosis through sIgM signaling. Transfectant with muscarinic M1 receptor in DT40 cells cannot undergo apoptosis upon carbachol stimulation despite the activation of the PtdIns pathway and calcium mobilization (Takata, M., and T. Kurosaki, unpublished result). An explanation is that in addition to activation of PtdIns pathway, PLC-γ2 might have another activity, yet undefined, which is required for the induction of apoptosis. It is also possible that sIgM-induced apoptosis requires another biochemical event(s) other than PLC-γ2 activation through sIgM signaling. An observation that simultaneous stimulation through sIgM and transfected M1 receptor failed to induce apoptosis in PLC-γ2–deficient cells (Takata, M., and T. Kurosaki, unpublished result) may support the first possibility.

The process of apoptosis is dependent on RNA and protein synthesis, leading to the concept of death genes that are responsible for the phenomena (72, 73). Recently, several genes implicated in apoptosis of different cell types have been identified, including c-*myc* (47, 74, 75), *nur77* (76, 77), *bcl-2* (45, 78–82), *bcl-x* (83), and *bax* (84). Thus, understanding the relationship between early signaling events and regulation of these genes should elucidate the mechanisms of sIgM-induced B cell apoptosis.

We thank M. Kurosaki for expert technical assistance, C.-L. Chen and M. Cooper for M4 mAb, and S. Nishikawa for critical reading of the manuscript.

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Received for publication 6 March 1995 and in revised form 28 April 1995.

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