Inhibition of anti-IgM induced translocation of Protein Kinase C \( \beta \)I inhibits ERK2 activation and increases apoptosis

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Running Title: Inhibition of PKC\( \beta \)I increases anti-IgM induced apoptosis.
SUMMARY

Expression of the COOH-terminal residues 179-330 of the LSP1\(^1\) protein in the LSP1\(^+\) B-cell line W10 increases anti-IgM or ionomycin induced apoptosis, suggesting that expression of this LSP1 truncate (B-LSP1) interferes with a Ca\(^{2+}\)-dependent step in anti-IgM signaling. Here we show that inhibition of Ca\(^{2+}\)-dependent cPKC isoforms with G\(\beta\)6976 increases anti-IgM induced apoptosis of W10 cells and that expression of B-LSP1 inhibits translocation of PKC\(\beta\)I but not of PKC\(\beta\)II or PKC\(\alpha\) to the plasma membrane. The increased anti-IgM induced apoptosis is partially reversed by over-expression of PKC\(\beta\)I. This shows that the B-LSP1 mediated inhibition of PKC\(\beta\)I leads to increased anti-IgM induced apoptosis. Expression of constitutively active PKC\(\beta\)I protein in W10 cells activates the MAPkinase ERK2, while expression of B-LSP1 inhibits anti-IgM induced activation of ERK2, suggesting that anti-IgM activated PKC\(\beta\)I is involved in the activation of ERK2 and that inhibition of ERK2 activation contributes to the increased anti-IgM induced apoptosis. Pull-down assays show that LSP1 interacts with PKC\(\beta\)I but not with PKC\(\beta\)II or PKC\(\alpha\) in W10 cell lysates, while \textit{in vitro} LSP1 and B-LSP1 bind directly to PKC\(\beta\)I. Thus B-LSP1 is a unique reagent that binds PKC\(\beta\)I and inhibits anti-IgM induced PKC\(\beta\)I translocation leading to inhibition of ERK2 activation and increased apoptosis.
INTRODUCTION

Many mouse and human B-lymphoma cell lines are susceptible to anti-IgM induced apoptosis (1-6). Multiple mIgM coupled signal transduction pathways such as increased $[\text{Ca}^{2+}]_i$, and production of ceramides and reactive oxygen species mediate the apoptotic effect of mIgM stimulation (7-9). However, anti-IgM treatment also activates potentially anti-apoptotic signaling pathways such as activation of PI3-kinase and its downstream target Akt/PKB or activation of PKC (10-12). Thus the outcome of anti-IgM signaling depends on a balance of pro-apoptotic and anti-apoptotic signals.

Activation and translocation of PKC by phorbol ester protects normal immature and mature mouse B-lymphocytes, the mouse B-lymphoma cell line WEHI-231 cells, human B-lymphoma cell lines and human B-CLL cells from anti-IgM induced apoptosis (1, 4, 13, 14). The precise PKC isoform involved in protection from anti-IgM induced apoptosis is not yet known. Evidence for a role of one or more of the Ca$^{2+}$-dependent cPKC isoforms -α, -βI, -βII or -γ in this protection comes from experiments showing that the protective action of phorbol esters on immature B-lymphocytes (13) is abrogated by a cPKC specific inhibitor. This inhibitor also renders mature B-lymphocytes susceptible to anti-IgM induced apoptosis (13), suggesting that anti-IgM induced activation of cPKC isoforms plays an important role in regulating susceptibility of different B-lymphocyte lineage cells to anti-IgM induced apoptosis.

The mouse Leukocyte Specific Protein 1 (LSP1) is a 330 amino acid residue intracellular protein expressed in B- and T- lymphocytes and in macrophages and neutrophils (15-19).
Transfection experiments using the LSP1+ B-lymphoma cell line WEHI-231/89 or a single cell subclone, W10, showed that expression of an LSP1 truncate containing residues 179-330 (designated B-LSP1) significantly increased the extent of apoptosis induced by anti-IgM or by ionomycin but not by sorbitol, nocodazole, C2 ceramide or H2O2 (20). Expression of B-LSP1 had no effect on anti-IgM induced growth arrest. Consistent with a role of LSP1 in the early induction phase of apoptosis, we found that after anti-IgM treatment, the number of cells showing loss of mitochondrial membrane potential ($\Delta \Psi_m$) increased faster in the B-LSP1 transfectant than in the parental cells (20). From these experiments we concluded that LSP1 regulates an early step in the induction of anti-IgM mediated apoptosis, downstream of the anti-IgM induced increase in $[Ca^{2+}]_i$, but upstream of the loss of $\Delta \Psi_m$. Given the protective role of the Ca$^{2+}$-dependent cPKC isoforms in anti-IgM induced apoptosis we asked whether the expression of B-LSP1 inhibits anti-IgM induced translocation of cPKC isoforms and if so whether this increases anti-IgM induced apoptosis by inhibiting the cPKC regulated activation of the MAPkinase ERK2.
EXPERIMENTAL PROCEDURES

Cell culture and apoptosis measurements. Cells were cultured in RPMI-1640 medium as described (20). The W10 cell line is a single cell subclone of the B-lymphoma cell line WEHI-231/89 and the TW10.1 cell line is a stable G418 resistant transfectant derived from W10 cells expressing the LSP1 truncate B-LSP1 containing LSP1 residues 179-330 (20). To over-express PKCβI, TW10.1 cells were co-transfected with the pcDNA3 vector containing a rat PKCβI cDNA and with the pBABE-puro vector, mixed in a 2:1 molar ratio. Cells were selected in 1 mg/ml G418 and 0.25 µg/ml puromycin. The constitutively active PKCβIΔNPS construct encoding rat PKCβI without the pseudosubstrate containing residues 1-30 was expressed following electroporation of W10 cells and selection in 1 mg/ml G418. Parental and transfected cells were cultured in 24-well plates with or without the addition of 5 µg/ml goat anti-mouse IgM (Sigma-Aldrich, Oakville, ON). Apoptosis was assessed using FACS analysis to determine the fraction of viable cells identified by their normal forward scatter / side scatter (FSC/SSC) profile after 72 hrs in culture or by determination of the fraction of cells containing subdiploid DNA 48 hrs. after the initiation of culture (20,21). Inhibitors were added either alone or 15 min before the addition of anti-IgM.

Cell fractionation and Western blotting. For PKC translocation experiments W10 or TW10.1 cells were washed twice in Hank’s BSS and resuspended at 25x10^6 cells/ml in HBSS. Three aliquots of 1 ml in microfuge tubes were then warmed at 37°C for 10 min in a waterbath. Goat anti-mouse IgM was added to a final concentration of 50 µg/ml to two tubes and incubation at 37°C was then continued. Cells from the third tube served as the unstimulated
controls and were recovered by centrifugation for 30 sec in a microfuge, washed once in cold HBSS and stored on ice. Anti-IgM stimulated cells were recovered after 5 or 20 min. of incubation. To prepare plasma membrane fractions, cells were resuspended for 30 min in cold hypotonic fractionation buffer (Buffer A, 5 mM Tris-HCl pH 7.4, 5 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA) containing a cocktail of protease inhibitors (22,23) and then disrupted by 50 strokes in a small Potter homogenizer. The lysates were cleared by centrifugation for 5 min at 500 x g. Supernatants were layered on top of 1.2 ml 1.2 M sucrose in buffer A and spun at 10,000 x g for 15 min to remove the majority of mitochondria. The lysates were then centrifuged at 100,000 x g for 60 min. The high speed pellet was designated as the plasma membrane fraction and was solubilized in buffer A, while the supernatant was designated as the cytoplasmic fraction. Protein concentrations were determined according to the Bradford method using reagents from BioRad (Oakville, ON).

Equal amounts of protein were separated on SDS-12.5% poly-acrylamide gels, transferred to PVDF membranes and analyzed by using antibodies specific for PKCα (Sigma-Aldrich, Oakville, ON), PKCβI or PKCβII (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Blots were developed with ECL reagent (Amersham-Pharmacia, Oakville, ON) followed by exposure to film or with SuperSignal West Femto reagent (Pierce Chemical Co., Rockford, IL) followed by imaging in a BioRad Fluor-Smax. Film exposures were converted to .tif files using a scanner and differences in band intensities were quantitated using Quantity One software from BioRad. Each gel contained a set of two-fold dilutions of a total cell lysate from W10 or TW10.1 cells to construct a standard curve used for quantitation of protein signals. Total lysates
were prepared by lysing $10^7$ W10 cells directly in 1 ml Laemmli sample buffer.

*Activation of ERK2.* Cells ($25 \times 10^6$ cells/ml) were warmed to 37°C for 10 min and then stimulated with 50 µg/ml anti-IgM with or without pretreatment with 0.25 µM Go6976 or 25 µM PD098059 for 15 min. Cells were harvested at different times after addition of anti-IgM, pelleted for 30 sec in a microfuge, lysed in 1x sample buffer and immersed in boiling water for 3 min. Phosphorylation of ERK2 was then analyzed on poly-acrylamide gels with a acrylamide : bisacrylamide ratio of 118:1 (24), followed by Western analysis with anti-ERK2 antibodies (Santa Cruz Biotechnology Inc, cat. # sc-154).

ERK2 kinase activity was measured in an *in vitro* immune kinase assay. Cells were harvested as above and resuspended in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.2 mM NaVO$_3$, 1mM DTT, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM PMSF). After incubation on ice for 15 min, the insoluble material was pelleted for 15 min at 13,000 rpm in a microfuge and ERK2 was precipitated from the soluble lysates by addition of 5 µg of anti-ERK2 antibody followed by incubation at 4°C. After 1 hr, 20 µl of protein G-agarose slurry (Pierce Chemical Co.) was added and incubation was continued for an additional 30 min. Protein G-agarose beads were recovered by centrifugation and washed 3 times in lysis buffer and once in kinase buffer (20 mM HEPES pH 7.2, 5 mM MgCl$_2$, 1 mM EGTA). To measure kinase activity, beads were resuspended in 30 µl of kinase buffer supplemented with 2 mM sodium vanadate, 5 mM β-2-mercaptoethanol, 7 µg myelin basic protein (MBP) and 5 µCi of $^{32}$P-γ-ATP (3000 mCi/mmole) and incubated for 15 min at 30°C. The reaction was stopped by adding 30 µl 3x Laemmli sample buffer prewarmed to 55°C,
followed by immersion in boiling water for 3 min. Phosphorylation of MBP was then analyzed on SDS-12.5% acrylamide gels and quantitated using a phosphoimager (Personal FX, BioRad).

**LSP1 / PKC interactions.** To probe for LSP1 / PKC interactions in cell lysates, 4x10^7 W10 cells were lysed in lysis buffer (20 mM Tris pH 7.5, 0.15 M NaCl, 5 mM EDTA) containing 0.5% NP-40 and a cocktail of protease inhibitors as described (22,23) and incubated with 1-2 µg of a GST-LSP1 fusion protein containing the intact LSP1 (residues 1-330). After 1 hr of incubation at 4°C, 10 µl of a 1:1 slurry of glutathione-Sepharose beads (Sigma-Aldrich) was added and incubated for an additional 30 min at 4°C. The beads were recovered by brief centrifugation in a microfuge, washed five times with 1 ml lysis buffer + 0.5 % NP-40 and the recovered proteins were analyzed by Western blotting. To determine binding of LSP1 with PKCβI in vitro, 1 µg of GST fusion protein containing LSP1 residues 1-330, 1-178 or 179-330 was mixed with 50 ng of recombinant human PKCβI (PanVera Corp., Madison, WI) in 250 µl 1xPKC buffer (20 mM Tris pH 7.5, 5 mM MgCl₂). After incubation for 1 hr. at 4°C, glutathione-Sepharose beads were added and the incubation was continued for an additional 30 min. Beads were recovered by centrifugation, washed four times in 1 ml of 1xPKC buffer and the amounts of bound PKCβI and recovered GST and GST fusion protein were analyzed by Western blotting.
RESULTS and DISCUSSION

Inhibition of cPKC isoforms increases anti-IgM induced apoptosis. Treatment of mature B-cells with a cPKC specific inhibitor renders these cells susceptible to anti-IgM induced apoptosis suggesting that anti-IgM activated cPKC plays a role in the protection of B-cells from anti-IgM induced cell death (13). To determine whether activated cPKC has a similar role in the B-lymphoma cell line W10 we treated these cells with anti-IgM in the presence or absence of Gö6976, a PKC inhibitor which preferentially inhibits the cPKC isoforms α, βI and βII (25,26). The extent of apoptosis was determined by measuring changes in the FSC/SSC profile of the cells at 72 hrs. (20,21). Treatment of W10 cells with 0.25 µM Gö6976 or with 5 µg/ml anti-IgM results in only a slight reduction of viable cells: 70.6 % of anti-IgM treated cells and 76.4 % of Gö6976 treated cells displays a normal FSC/SSC profile after 72 hrs. of culture (fig. 1). In contrast the addition of both agents reduces the number of cells with a normal FSC/SSC profile to 10.4 %. The addition of Gö6976 could be delayed for at least 20 min after addition of anti-IgM which shows that the protective action of cPKC depends on a relatively late event and that activation of cPKC immediately following anti-IgM stimulation is not sufficient to protect W10 cells from anti-IgM induced apoptosis (not shown). The combined effect of Gö6976 and anti-IgM on apoptosis of W10 cells is similar to the effect of expressing B-LSP1 as only 16.8 % of the anti-IgM treated TW10.1 cells display a normal FSC/SSC profile after 72 hrs. To confirm that cell death occurs by apoptosis we determined the number of cells containing subdiploid DNA after 48 hrs of culture. W10 cells treated with anti-IgM or with Gö6976 contain 20.8 % or 20.7 % cells with subdiploid DNA respectively, while W10 cells treated with both agents contain 50.1 % cells with subdiploid DNA. Again the effect of expressing B-LSP1 is
similar to the effect of treatment with Gö6976 as 55.2% of anti-IgM treated TW10.1 cells contain subdiploid DNA (not shown). Since the cPKCγ isoform is not expressed in these cells (27) these results suggest that the anti-apoptotic effect of activated cPKC is due to activation of one or more of the PKCα, βI or βII isoforms.

Expression of LSP1 residues 179-330 inhibits translocation of PKCβI to the plasma membrane. Increased anti-IgM induced apoptosis was evident after treatment of W10 cells with Gö6976 or after transfection with B-LSP1 containing LSP1 residues 179-330. To determine whether expression of B-LSP1 inhibits the activation of cPKC, we measured the extent of anti-IgM induced translocation of PKCα, βI, and βII to the plasma membrane fractions of the W10 cells and the transfectant TW10.1. Translocation of PKC is often used as a measure of activation (28). Plasma membrane fractions were prepared from unstimulated cells and from cells stimulated at 37°C with anti-IgM for 5 min or 20 min. Equal amounts of plasma membrane protein were analyzed for the presence of PKCα, -βI and -βII by Western blotting. All three PKC isoforms tested translocated to the plasma membrane after treatment of W10 cells with anti-IgM (fig. 2). Translocation was evident after 5 min of stimulation and did not change significantly for the next 15 min. Translocation of PKCα and PKCβII in TW10.1 cells did not differ significantly from that measured in W10 cells. Interestingly, translocation of PKCβI was significantly inhibited in TW10.1 cells, when measured 5 min or 20 min after addition of anti-IgM. We conclude from these data that expression of B-LSP1 specifically inhibits anti-IgM induced PKCβI translocation to the plasma membrane.

To determine whether the increased anti-IgM induced apoptosis of TW10.1 cells is due to the inhibition of PKCβI activation, we transfected TW10.1 cells with an expression vector
encoding intact rat PKCβI and selected four colonies in which the expression levels of PKCβI were 2–3 times higher than in the untransfected TW10.1 cells. We also isolated four control colonies that express the puromycin resistance gene but have no increased levels of PKCβI. Stimulation with anti-IgM showed that over-expression of PKCβI rendered the TW10.1 cells less susceptible to anti-IgM induced apoptosis (fig.1). While only 16.8% of TW10.1 cells or 17.1% of the puromycin resistant control colonies (not shown) were viable after 72 hrs of anti-IgM stimulation, 35.4% of PKCβI over-expressing TW10.1 transfectants remained viable. This shows that the B-LSP1 mediated inhibition of PKCβI contributes significantly to the B-LSP1 mediated increase in anti-IgM induced apoptosis of TW10.1 cells. The partial reversal may be related to the level of over-expression of PKCβI. Alternatively it may indicate that expression of B-LSP1 also affects anti-IgM induced apoptosis through mechanisms that do not involve PKCβI activation.

Inhibition of PKCβI translocation inhibits anti-IgM induced ERK2 activation. To determine whether the specific inhibition of anti-IgM induced translocation of PKCβI affects a known cPKC regulated, anti-IgM stimulated signaling pathway we measured the anti-IgM induced activation of the MAPkinase ERK2 in W10 and TW10.1 cells. Activation of ERK2 is associated with survival in many cell types (29,30) and results in figure 1 show that this is the case in W10 cells as well. Treatment of W10 cells with 25 μM PD098059, an inhibitor specific for MEK1 (31), the direct activator of ERK2, does not significantly affect cell viability as 89.1% of W10 cells displayed a normal FSC/SSC profile after 72 hrs of culture. However, in cultures treated with PD098059 and 5 μg/ml anti-IgM only 34.3% of cells were viable, showing that anti-IgM induced activation of ERK2 protects W10 cells from anti-IgM induced apoptosis.
Anti-IgM stimulation activates ERK2 in a PKC independent manner through the RAS/Raf-1/MEK1 pathway (12,32,33) and in a PKC dependent manner through activation of Raf-1 (34-36). Different PKC isoforms can contribute to Raf-1 activation when over-expressed in COS cells (37) but the specific PKC isoform involved in anti-IgM induced Raf-1/MEK1/ERK2 activation in B-cells is not yet known. Figure 3A shows that treatment of W10 cells with 0.25 µM Gö6976 or with 25 µM PD098059, significantly inhibits anti-IgM induced ERK2 activation as measured by the appearance of phosphorylated ERK2 which has a slightly lower mobility on SDS-PAGE than the non-phosphorylated form of ERK2. These results confirm that in W10 cells activated cPKC isoforms contribute to the activation of ERK2 and that activation of ERK2 depends on active MEK1. It is interesting to note that the affect of Gö6976 is more pronounced after 20 min than after 2 min of anti-IgM stimulation, while the MEK1 inhibitor PD098059 acts equally well at both time points. Increasing the pre-incubation time for Gö6976 to 35 min gave a similar result (not shown). This suggests that the early activation of MEK1/ERK2 by anti-IgM stimulation is less dependent on activation of cPKC, while at later times activated cPKC contributes significantly to ERK2 activation. The early activation of ERK2 activation may be more dependent on activated RAS.

We determined the involvement of PKCβI in anti-IgM induced ERK2 activation by two approaches. First W10 cells were transfected with pcDNA3 based expression vector encoding a constitutively active PKCβI protein (PKCβIΔNPS, lacking the N-terminal pseudosubstrate region) and selected two colonies expressing the PKCβIΔNPS protein. Two G418 resistant colonies that do not express PKCβIΔNPS were selected as control cells. Figure 3B shows that ERK2 activity is 4-5 times higher in the PKCβIΔNPS expressing transfectants than in the
control cells, showing that activation of PKCβI leads to activation of the ERK2 pathway.
Second, we analyzed ERK2 activation in W10 and TW10.1 cells in which the anti-IgM induced translocation of PKCβI is inhibited. ERK2 activation was measured 2, 10 and 20 min. after anti-IgM treatment using the decreased mobility of phosphorylated ERK2 on SDS-PAGE as a read-out (fig. 3C) or by measuring ERK2 activity in an immune kinase assay (fig. 3D,E). In W10 cells, ERK2 is activated efficiently after 2 min. of anti-IgM stimulation and does not change significantly over the next 15 min. The extent of activation of ERK2 in TW10.1 cells is similar to that found in W10 cells when measured 2 or 10 min after stimulation but after 20 min is significantly less than in W10 cells. These data show that inhibition of cPKC with Gö6976 or inhibition of PKCβI by expression of B-LSP1 both inhibit only the late but not the early activation of ERK2 by anti-IgM. This is strong evidence that anti-IgM induced activation of PKCβI contributes significantly to the late activation of ERK2.

Given that inhibition of ERK2 by PD098059 increases the extent of anti-IgM induced apoptosis, we suggest that the inhibition of ERK2 activation in TW10.1 cells contributes to the increase in anti-IgM induced apoptosis. The designation of ERK2 as an anti-apoptotic protein does not agree with a report showing that inhibition of anti-IgM induced activation of ERK2 in WEHI-231 cells protects from anti-IgM induced apoptosis (38). These discordant findings may be related to the different methods used to inhibit ERK2 activation. Whereas we established a protective role for ERK2 using the MEK1 inhibitor PD098059 to inhibit ERK2, the pro-apoptotic role of ERK2 was established using expression of the phosphatase MKP-1 to inhibit ERK2. However, this phosphatase is not specific for ERK2 and inhibits other members of the MAPkinase family, p38 and SAPK/JNK1 as well (39).
PKCβI interacts with LSP1 residues 179-330. We used pull-down experiments to determine whether LSP1 interacts with PKCβI. A GST-fusion protein containing the intact LSP1 or the GST protein was mixed with NP-40 soluble lysates from W10 cells. After incubation for 1 hr. the GST protein and the LSP1/GST fusion proteins were recovered using glutathione-Sepharose beads and analyzed by Western blotting. Using this protocol PKCβI but not PKCα or PKCβII were recovered from lysates when mixed with the LSP1 / GST fusion protein. No detectable amounts of PKCα, βI or βII were recovered using the GST protein, indicating a specific interaction of LSP1 with PKCβI (fig. 4A). To determine whether PKCβI and LSP1 interact directly we performed in vitro binding assays using human recombinant PKCβI and GST-fusion proteins containing different LSP1 sequences. One µg of GST or GST fusion protein was incubated with 50 ng of recombinant human PKCβI. Figure 4B shows that PKCβI interacts preferentially with intact LSP1 or with the COOH-terminal domain residues 179-330. In replicate experiments binding to the NH2-terminal residues 1-178 is not significantly higher than binding to the GST protein alone indicating that the preferential binding site or sites for PKCβI are located between LSP1 residues 179-330.

We do not as yet know why expression of LSP1 residues 179-330 in the LSP1+ TW10.1 cells inhibits translocation of PKCβI, but we propose that the endogenous, intact LSP1 sequesters inactive PKCβI to a cytosolic localization and that in response to anti-IgM generated signals the LSP1 / PKCβI complex dissociates, allowing for the translocation of PKCβI. The release of PKCβI from B-LSP1 in response to anti-IgM stimulation may be less efficient, leading to inhibition of PKCβI translocation. LSP1 is a Ca^{2+}-binding protein and contains two
putative Ca$^{2+}$-binding EF-hand motifs near the NH$_2$-terminus (16). Thus binding of Ca$^{2+}$ to the NH$_2$-terminal domain may result in a structural change in the COOH terminal domain leading to dissociation of PKC$\beta$I. Since both EF-hand motifs are absent from B-LSP1, suggesting that B-LSP1 does not bind Ca$^{2+}$, the B-LSP1 / PKC$\beta$I complex may not dissociate after the anti-IgM induced increase in [Ca$^{2+}$]$_i$ thereby inhibiting the movement of PKC$\beta$I to the plasma membrane. Alternatively, since the NH$_2$-terminal domain of LSP1 contains a phosphorylation site for casein kinase II (40) and several putative PKC phosphorylation sites, it is possible that the binding of PKC$\beta$I to LSP1 residues 179-330 is regulated by phosphorylation of specific sites in the LSP1 NH$_2$ terminal domain. Thus, LSP1 may protect from apoptosis only in response to certain signals such as increased [Ca$^{2+}$]$_i$. Apoptosis induced by sorbitol, nocodazole, C$_2$ ceramide or H$_2$O$_2$ is not regulated by LSP1 (20) possibly because these signals do not lead to dissociation of PKC$\beta$I from LSP1.

The study of PKC isoform specific functions is hampered by the lack of isoform specific activators or inhibitors. Our data suggest that B-LSP1 is a unique reagent that specifically inhibits the anti-IgM induced activation of PKC$\beta$I. Experiments to determine whether B-LSP1 also inhibits PKC$\beta$I translocation in response to other Ca$^{2+}$-generating signals such as ionomycin or anti-CD20 stimulation are currently underway. We have used expression of B-LSP1 to inhibit anti-IgM induced translocation of PKC$\beta$I in a transformed B-cell line, which significantly impacted on the susceptibility of these cells to anti-IgM induced apoptosis. Many B-lymphoma cell lines are susceptible to anti-IgM induced apoptosis, a characteristic which
forms the basis for using anti-Ig antibodies as therapy for B-lymphoma in vivo (41,42). Our results suggest that the efficiency of anti-Ig therapy may be enhanced by the concomitant inhibition of PKCβI.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute of Canada with funds from the Canadian Cancer Society and from the Cancer Research Society, Inc. We thank Atri Persad for technical help and Dr. C. Whiteside, University of Toronto, for a kind gift of anti-PKC antibodies.
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**FOOTNOTES**

1  The abbreviations used are : LSP1, Leukocyte Specific protein 1; mIgM, membrane immunoglobulin M; cPKC, conventional Protein Kinase C.
FIGURE LEGENDS

Fig. 1. Inhibition of cPKC or MEK1 increases anti-IgM induced apoptosis. W10 cells, TW10.1 cells (10.1) and PKCβI over-expressing TW10.1 transfectants (10.1/βI) were cultured in 24-well plates as described with or without anti-IgM (5 µg/ml) or the PKC inhibitor Gö6976 (0.25 µM) or the MEK1 inhibitor PD098059 (25 µM) as indicated. The inhibitors were added 15 min before addition of anti-IgM and the percentage of viable cells were identified by their normal FSC/SSC profile after 72 hr of culture.

Fig. 2. Expression of LSP1 truncate 179-330 inhibits anti-IgM induced translocation of PKCβI. A, Plasma membrane fractions were prepared from unstimulated W10 and TW10.1 cells and from cells stimulated for 5 or 20 min with 50 µg/ml anti-IgM. Equal amounts of plasma membrane protein were then separated on SDS-12.5% acrylamide gels and analyzed for PKCβI or PKCβII. Lanes 1-4 are two-fold dilutions of a total lysate from W10 cells to construct a standard curve used to quantitate the amount of PKCβI or PKCβII in the different plasma membrane fractions (lanes 5-10). A 79 Kd molecular mass marker is indicated at the left. The results shown are of one experiment typical of four experiments performed. B, results are presented as average fold increase over unstimulated cells calculated from three or four experiments performed. r, W10 cells. p, TW10.1 cells. Differences were tested for statistical significance using the Student’s t-test. **, P<0.01.

Fig.3. Inhibition of PKCβI leads to inhibition of ERK2 activation. A, W10 cells were stimulated with anti-IgM alone (top row) or with anti-IgM and the cPKC inhibitor Gö6976 (middle row) or with anti-IgM and the MEK1 inhibitor PD098059 (bottom row). Cells were
harvested just before or 2 or 20 min. after addition of anti-IgM and lysed in sample buffer. ERK2 phosphorylation was determined by Western blotting. Phosphorylated ERK2 is indicated with a * and has a slightly lower mobility during PAGE than the non-phosphorylated ERK2. B, W10 were transfected with the constitutively active PKCβIΔNPS construct. Two control transfectants and two transfectants over-expressing PKCβIΔNPS (approximately 3-fold compared with the endogenous PKCβI, not shown) were assayed for ERK2 activity using an *in vitro* immune kinase assay with MBP as substrate. Both PKCβIΔNPS expressing transfectants contained 4-5 fold more ERK2 activity than the control cells. C, anti-IgM induced ERK2 activation of W10 and TW10.1 cells. *, phosphorylated ERK2. D, ERK2 activity was measured using an immune kinase assay. The results shown in A-D are of one experiment typical for three experiments performed. E, quantitative representation of anti-IgM induced ERK2 activation as measured by immune kinase assay. Results are expressed as fold increase +/- S.E.M. of MBP phosphorylation in anti-IgM stimulated samples over unstimulated controls. Solid bars, W10 cells. Open bars, TW10.1 cells.

**Fig. 4. LSP1 interacts with PKCβI.** A, pull-down experiments were performed by incubating GST or a GST fusion protein containing LSP1 residues 1-330 with NP-40 soluble lysates from W10 cells as described and proteins associated with GST or the GST-LSP1 fusion protein were analyzed for the presence of PKCα, βI and βII by Western blotting. TL, total lysate prepared by lysis of W10 cells directly into SDS-PAGE sample buffer. The position of a 73 Kd molecular mass marker is indicated at the left. B, recombinant human PKCβI (50 ng) was incubated with 1 µg GST or GST fusion proteins containing LSP1 residues 1-330, 1-178 or 179-330. GST and GST fusion proteins were recovered on glutathione-Sepharose beads and
analyzed for the presence of PKCβ1 (top panels) and GST or GST fusion proteins (bottom panels) by Western blotting. In the bottom panels only that portion of the Western blots containing the GST or GST fusion protein is shown.
Inhibition of anti-IgM induced translocation of protein kinase C \( \beta \)I inhibits ERK2 activation and increases apoptosis

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*J. Biol. Chem.* published online May 1, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M103883200](http://doi.org/10.1074/jbc.M103883200)

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