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Suppression of apoptosis by v-ABL protein tyrosine kinase is associated with nuclear translocation and activation of protein kinase C in an interleukin-3-dependent haemopoietic cell line

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SUMMARY

We previously demonstrated that activation of v-ABL protein tyrosine kinase resulted in suppression of apoptosis following interleukin-3 removal using an interleukin-3-dependent haemopoietic cell line transfected with a temperature-sensitive mutant of the v-*abl* oncoprotein (IC.DP). Cellular signalling events associated with the activation of v-ABL included increased levels of *sn*-1,2-diacylglycerol, an activator of protein kinase C. Calphostin C, a PKC inhibitor, restored apoptosis to interleukin-3-deprived IC.DP cells expressing active v-ABL. However, chronic exposure to the phorbol ester, 12-*O*-tetradecanoyl phorbol 13-acetate to downregulate protein kinase C did not attenuate the survival of IC.DP cells expressing active v-ABL. Translocation of a classical protein kinase C isozyme(s) to the nuclear fraction was observed 6 hours after activation of v-ABL, when nuclear protein kinase C activity was increased approximately 2-fold. The protien kinase C isozyme responsible, which was only partially downregulated by 12-*O*-tetradecanoyl phorbol 13-acetate, was identified as protein kinase C β_{II}. This translocation of protein kinase C β_{II} to the nucleus was inhibited by calphostin C. Taken together, these results suggest that nuclear translocation and activation of PKCβ_{II} may play a role in v-ABL-mediated suppression of apoptosis.

Key words: apoptosis, survival signal, protein kinase C, v-ABL, haemopoietic cell line, interleukin-3 withdrawal

INTRODUCTION

Chronic myeloid leukaemia (CML) is a haematological disease characterised by the Philadelphia chromosomal rearrangement t(9:22), (q34,q11) which generates the chimeric *bcr-abl* gene. The protein encoded by *bcr-abl* is thought to play a key role in the development of CML (Daley et al., 1990; Elefanty et al., 1990; Kelliher et al., 1990) and resembles v-ABL, the transforming agent of the Abelson murine leukaemia virus, in that they are both constitutively active non-receptor tyrosine kinases. Like the BCR-ABL protein tyrosine kinase (PTK), v-ABL produces a CML-like disease in mice (Kelliher et al., 1990).

Clinically, CML patients in chronic phase have an increased number of immature blast cells in the bone marrow and peripheral blood, and the mature myeloid cell number is also greatly increased in the bloodstream. This is due to the partial uncoupling of processes during haemopoiesis which normally function to maintain a homeostatic blood cell supply: haemopoiesis is controlled by a range of haemopoietic growth factors (HGFs), which regulate stem cell self-renewal, differentiation to mature blood cell types and the balance between proliferation and cell death within the bone marrow and blood. In vitro, removal of HGFs results in cell death via apoptosis (Williams et al., 1990; Koury, 1992).

We have previously demonstrated that activation of v-ABL suppressed apoptosis induced both by the withdrawal of the haemopoietic growth factor, interleukin-3 (IL-3), and by the addition of the antimetabolite hydroxyurea (used to treat CML), in the IL-3-dependent cell line, IC.DP (Evans et al., 1993; Chapman et al., 1994). This suppression of apoptosis provides a potential mechanism for leukaemic population expansion in vivo and may explain the increasing degree of drug resistance observed during the chronic phase of the disease prior to the onset of the generally fatal blast crisis (Gerson et al., 1988; Hincks et al., 1992).

Our previous studies on the effects of activation of v-ABL on cell fate in vitro were performed using a mutant, temperature-sensitive, form of the v-*abl* oncoprotein, which is active as a PTK at 32°C and inactive at 39°C. Apoptotic suppression

mediated by v-ABL in IC.DP cells following IL-3 withdrawal in serum-free conditions occurred in the absence of cell proliferation, allowing us specifically to examine the signal transduction pathways whereby v-ABL suppresses apoptosis. We have previously demonstrated that v-ABL activation was followed by phospholipid hydrolysis to generate choline phosphate (ChoP) and the second messengers inositol 1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG), which is a physiological activator of protein kinase C (PKC). In addition, v-ABL activation stimulated an increase in glucose transport (On et al., 1994) and cellular alkalinisation. The increase in intracellular pH was mediated by the amiloride-sensitive sodium hydrogen antiport and was attenuated when cells were preincubated and chronically exposed to the PKC-interactive phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (Owen et al., 1993). The nature and kinetics of the above events with respect to the survival advantage conferred by v-ABL activation strongly suggest that PKC may play a role in suppression by v-ABL of apoptotic cell death.

PKC is a family of at least ten structurally related serine/threonine kinases whose activation plays a key role role in the regulation of fundamental cell processes such as growth and differentiation (Hug and Sarre, 1993). Precedents now exist for a role for PKC in the modulation of apoptosis: but this role is ill defined and was inferred from experiments where apoptosis levels were assessed after treating cells with compounds known to enhance and/or inhibit PKC activity (Dive et al., 1992a). Most previous studies employed non-specific kinase inhibitors and the phorbol ester TPA (McConkey et al., 1989; Rodriguez-Tarduchy and Lopez-Rivas, 1989; Perotti et al., 1990; Rajotte et al., 1992). The results were contradictory, even in the same cell type, and the role of PKC in the modulation of apoptosis demands more rigorous assessment.

The apparent conflict in the literature regarding the role of PKC in apoptosis regulation is undoubtedly also due in part to the existence of multiple isozymes of PKC; these include classical Ca^{2+}-dependent cPKCs α, β_I, β_{II}, γ, novel Ca^{2+}-independent nPKCs δ, ε, η and θ, and atypical phorbol ester-insensitive isozymes PKCζ and λ (Hug and Sarre, 1993). The PKC isozymes show differential substrate specificities, co-factor sensitivities and differential expression with cell type and cell status. These data have been proposed to suggest that PKC isozymes have specific roles in cell regulation and function (Farago and Nishizuka, 1990). Furthermore, TPA is known to differentially modulate isozymes of PKC (Hug and Sarre, 1993). Interpretation of data gained with TPA, regarding the role of PKC isozymes in apoptosis regulation, is therefore difficult. To date, there is little evidence regarding the role of specific PKC isoforms in the signal transuction pathways which modulate apoptosis. The few studies that have considered PKC isozymes, have implicated the involvement of PKCβ in apoptosis. Knox et al. (1993) showed that expression of PKCβ and PKCα was elevated in apoptotic tonsil epithelial cells, the β isoform involved was not determined. Recent studies from our group on myelomonocytic U937 cells showed that activation of PKCβ_I was associated with initiation of apoptosis (Pongracz et al., 1993, 1994).

Current models for the activation of PKC, at least cPKCs, include binding of Ca^{2+} to the C2 region and subsequent translocation to cell membranes (reviewed by Bell and Burns, 1991). In these studies, we have used the IC.DP cell line, deprived of IL-3, to determine whether or not PKC is translocated and therefore activated, subsequent to the activation of v-ABL, and thereby to identify the PKC isoforms which might be involved in the suppression of apoptosis.

MATERIALS AND METHODS

Materials

Acridine Orange and Hoechst 33342 were purchased from Molecular Probes Inc., Oregon, USA. PKC isozyme-specific anti-peptide polyclonal antisera were obtained from Gibco, UK. Peroxidase-labelled donkey anti-rabbit antibodies, Hybond C nitrocellulose membrane and the enhanced chemiluminescence (ECL) kit were from Amersham, UK. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody was supplied by the Binding Site Limited, Birmingham, UK. Plasticware was obtained from Costar, UK. All other chemicals were of analytical grade and were purchased from Sigma, UK or BDH, UK. IC.DP cells were a gift from Dr Jean Wang, University of California, USA.

Cell culture

IC.DP and the parental IC2.9 cells were routinely cultured as described previously (Evans et al., 1993). Briefly, cells were cultured at 37°C in Fischer's medium supplemented with 10% horse serum and 3% IL-3-containing medium conditioned by X63-Ag-653 cell line transfected with the IL-3 gene (mIL-3 CM) (Karasuyama and Melchers, 1988). For experiments investigating the effects of temperature switching to activate v-ABL, cells were generally maintained at 39°C for 18 hours preceding a temperature switch to 32°C.

Measurement of apoptosis

Acridine Orange (10 μg/ml in phosphate buffered saline) staining was used to identify apoptotic cells via changes in nuclear morphology as previously described (Gregory et al., 1991).

Subcellular fractionation

Cells were washed three times in PBS, resuspended at 3×10^7 cells/ml in a hypotonic buffer (50 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 5 mM $MgSO_4$, 1 mM dithiothreitol, 0.1 mM $NaVO_3$, 20 mM NaF, 200 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 0.02% NP-40) and swollen on ice for 20 minutes. The cells were then disrupted to release the nuclei by repeated passage through a needle (25 G). Samples were centrifuged at 1,000 ***g*** for 1 minute at 4°C to yield a 'nuclear' pellet and supernatant containing the 'cytosol/membrane' fraction. The supernatants were ultracentrifuged (100,000 ***g***, 60 minutes, 4°C) to separate the 'membrane' pellet from the 'cytosolic' supernatant fractions. The nuclear pellet was resuspended and spun through a 50% sucrose cushion to remove contaminating membrane fragments. Biochemical markers for the resultant subcellular fractions were analysed: lactate dehydrogenase was used to identify cytosol, leucine aminopeptidase was used to identify membranes (Roncari and Zeuber, 1968) and the DNA:protein ratio was assessed to identify the nuclear fraction (Divecha et al., 1991). Lactate dehydrogenase was assayed in 55 mM KPO_4 buffer pH 7.4, containingh 1.4 mM NADH and 1.8 mM sodium pyruvate by monitoring absorbance at 340 nm. DNA was quantified using Hoechst 33342 (Dive et al., 1992b) and protein was measured using the Bradford method (Bradford, 1976). In all experiments described below, cross-contamination of subcellular fractions was found to be less than 5%.

PKC activity measurements

PKC activity was assayed using histone III-S as the substrate. The method was essentially that previously described by Basu et al. (1990), modified to allow assay of both Ca^{2+}-dependent and inde-

pendent PKC isozymes (Borner et al., 1992). The reaction mixture (50 μl) contained 20 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 10 mM $Mg(CH_3COO)_2$, 1.25 μg phosphatidylserine, 0.125 μg *sn*-1,2-diolein, 400 μM $CaCl_2$, 100 μM EGTA, 20 μg histone III-S and 24 μM ATP containing 0.5 μCi [τ-^{32}P]ATP. Basal activity was that obtained in the presence of 5 mM EGTA without the co-factors, phosphatidylserine (PS), DAG and Ca^{2+}. Activity of Ca^{2+}-independent, nPKC and atypical PKC enzymes were assayed in the presence of phosphatidylserine and DAG with 5 mM EGTA replacing Ca^{2+}.

Immunohistochemical analysis of PKC isozyme localisation using confocal microscopy

Cytospins of samples were prepared using a Shandon cytospin centrifuge (500 rpm, 3 min). The cells were air dried for 1 hour prior to fixation in ice-cold methanol. Immunocytochemical staining was performed using a panel of affinity-purified PKC isozyme-specific anti-peptide polyclonal antisera to PKCα, β_I, β_{II} δ, ε and ζ (Gibco). The suitability of the anti-PKCα, δ and ε antisera for immunochemistry and their isozyme specificity have already been reported (Knox et al., 1993). In these studies the anti-PKCβ_I, β_{II} and ζ antisera were also found to be isozyme selective; in immunostaining, fluorescence could be titered out to an end point and was therefore not non-specific, and staining was removed by the appropriate immunogenic peptide. The secondary antibody used was a FITC-conjugated sheep anti-rabbit IgG antibody. Nuclei were counterstained with propidium iodide and fluorescence fading was retarded by 2.4% 1,4-diazobicyclo-(2,2,2)-octane (DABCO) in 80% (w/v) glycerol (Johnson et al., 1982). Samples were analysed using a Biorad MRC 500 confocal microscope.

Western blotting

Samples were resolved by SDS-PAGE (7.5% acrylamide) prior to transfer onto a nitrocellulose membrane. The membranes were probed using one of a panel of isozyme-specific anti-peptide anti-PKC polyclonal antibodies as the primary antibody. The secondary antibody was donkey anti-rabbit coupled to horseradish peroxidase. Immunoreactive bands were detected using the ECL method. The specificity of the primary antibodies was confirmed by abolition of an immunoreactive band of appropriate molecular mass (76-80 kDa for PKCα, β_{II}, δ ζ and 88 kDa for PKCε) was abolished in the presence of the appropriate immunogenic peptide.

Immunoprecipitation

Cells were lysed in buffer (50 mM Tris-HCl, pH 7.5, 5 mM EGTA) containing 1% Triton X-100, 0.15 M NaCl and protease inhibitors (1 mM PMSF, 5 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin) and sonicated for 10 seconds on ice (Soniprobe V). Lysates were cleared by centrifugation and supernatants pre-cleared by incubation with Protein A-Sepharose for 30 minutes at 4°C. Protein A-Sepharose was then removed and the supernatant incubated with the anti-PKCβ_{II} isozyme specific antibody (overnight, 4°C), followed by Protein A-Sepharose for 2 hours at 4°C. Immunoprecipitates were then isolated by centrifugation and boiling of Protein A-Sepharose in SDS sample buffer. Samples were resolved by SDS-PAGE, equivalently loaded for protein (50 μg/lane). Proteins were visualised by staining of gels with Brilliant Blue colloidal G.

RESULTS

PKC plays a role in v-ABL-mediated suppression of apoptosis

We have previously demonstrated that activation of v-ABL in IC.DP cells stimulated an increase in phospholipid breakdown to generate the second messenger DAG (Owen et al., 1993).

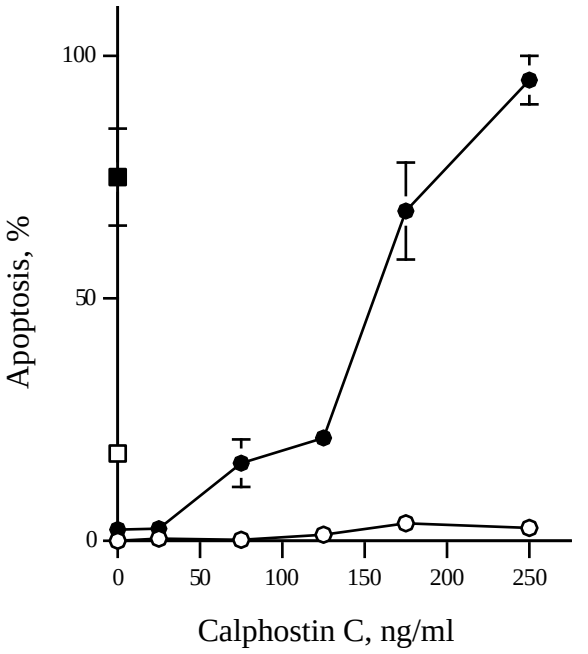

Fig. 1. Effect of treatment with calphostin C on the kinetics of v-ABL PTK-mediated suppression of apoptosis in IL-3-deprived IC.DP cells. Cells were pretreated with increasing concentrations of calphostin C (0-250 ng/ml) for 15 minutes prior to maintenance at 39°C (squares) or activation of v-ABL by temperature switching to 32°C (circles). Cell death was assessed 24 hours (black square, black circles) and 48 hours (white square, white circles) later by Acridine Orange staining and fluorescence microscopy to detect nuclear changes characteristic of apoptotic cells. Percentage apoptosis was calculated from the proportion of cells exhibiting apoptotic morphology. Results shown are mean ± s.e.m., *n*=3 experiments.

Since DAG is a physiological activator of the serine/threonine kinase PKC, the possible role of PKC activation in the suppression of apoptosis by v-ABL was investigated. We first examined whether or not compounds which modulate PKC activity could restore apoptosis in IL-3-deprived IC.DP cells at 32°C (i.e. under conditions where the v-ABL PTK was active). Treatment with the PKC inhibitor, calphostin C resulted in concentration-dependent inhibition of the suppression of apoptosis by v-ABL (Fig. 1). The IC_{50} value for this inhibition was 115 nM ± 8 (s.e.m. *n*=3), which is consistent with inhibition of PKC by calphostin C in in vitro kinase assays (Tamaoki, 1991). There was no evidence that calphostin C at concentrations up to 160 nM affected the increase in tyrosine phosphorylation observed in IC.DP cells subsequent to activation of v-ABL PTK (Owen et al., 1993), as demonstrated by western blotting analysis using an anti-phosphotyrosine antibody (data not shown). Experiments were also performed using the phorbol ester TPA, which acutely activates, and chronically downregulates, PKC (Castagna et al., 1982; Stabel et al., 1987). TPA (166 nM), was added to IC.DP cells for 4 hours prior to activation of v-ABL PTK by temperature switch to 32°C and was present in the culture following removal of IL-3 and throughout the time course of suppression of apoptosis by v-ABL (0-72 hours) (Fig. 2A). The levels of apoptosis observed were completely unaffected by the presence of TPA (i.e. v-ABL still suppressed apoptotic cell

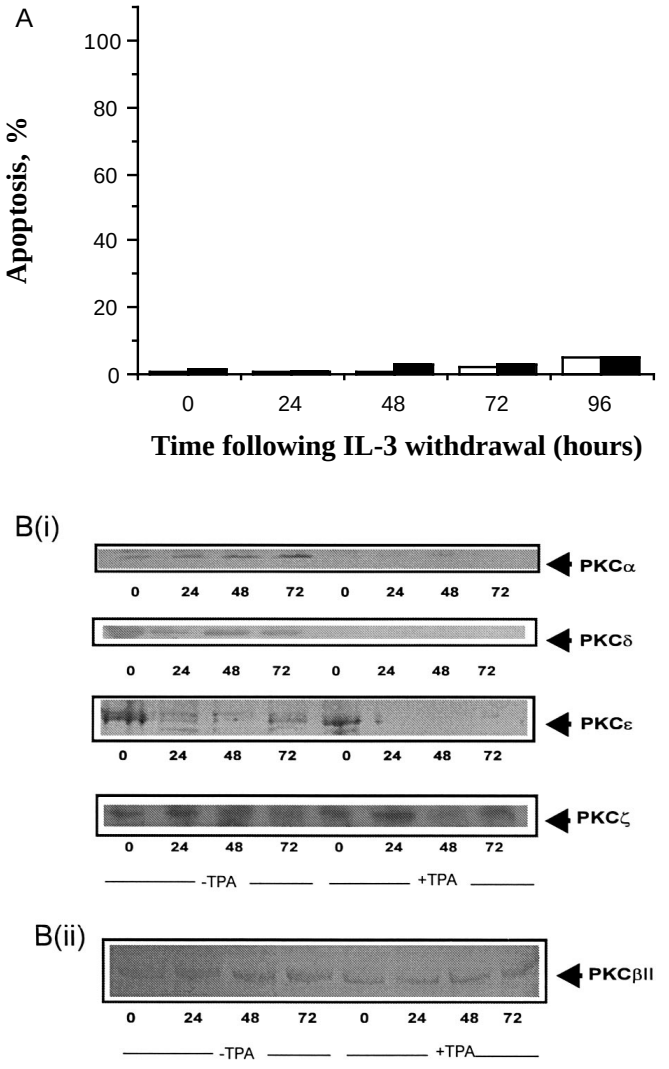

Fig. 2. Effect of TPA treatment on kinetics of v-ABL-mediated apoptotic suppression. (A) IC.DP cells were pretreated with TPA (166 nM, black bars) or TPA diluent DMSO (0.05% v/v, white bars) for 4 hours prior to activation of v-ABL PTK by temperature switch to 32°C. Cell death was assessed 24, 48, 72 and 96 hours later by acridine orange staining and fluorescence microscopy to detect nuclear morphology changes characteristic of apoptotic cells. Percentage apoptosis was calculated from the proportion of cells exhibiting apoptotic morphology. Results shown are mean ± s.e.m, n=3 experiments. (B) Effect of treatment with TPA on the levels of PKC isozymes in IL-3-deprived IC.DP cells with activated v-ABL PTK. (i) Western blot analysis using PKCα, δ, ε and ζ isozyme-specific antibodies; (ii) immunoprecipitation of PKCβII for IC.DP cells from the time of initial temperature switch from 39°C to 32°C (time 0) to 72 hours following v-ABL PTK activation.

death). In view of the differential susceptibility of the various PKC isozymes to downregulation by TPA (Hug and Sarre, 1993), western blot or immunoprecipitation analysis of samples prepared from IC.DP cells cultured at 32°C in the presence and absence of TPA was performed using a panel of isozyme-specific anti-PKC antibodies. IC.DP cells expressed several cPKC and nPKC isozymes: cPKCα and βII, nPKCδ, ε and the atypical PKCζ (Fig. 2B). The amounts of PKCα and PKCε were downregulated to undetectable levels by TPA by 24 hours, whilst PKCβII and PKCζ levels were reduced compared to the original level, but not completely downregulated. These results suggest the PKCβII and/or PKCζ as candidates for activation by v-ABL to suppress apoptosis, since they are not completely downregulated by TPA. Cycloheximide (10 μM) did not inhibit v-ABL-mediated supression of apoptosis (6.1±1.3% non-viable IC.DP cells 72 hours following IL-3 removal in the presence of 10 μM cycloheximide compared to 10.2±1.8% for the untreated control cells). These data suggest that de novo protein synthesis is not required for apoptotic supression by v-ABL. We have studied the effect of v-ABL activation on the subcellular localisation of PKCβII and PKCζ to investigate their involvement in the suppression of apoptosis.

Table 1. The effect of v-ABL activation on total cellular levels of PKC isozymes in IC.DP cells measured by immunohistochemistry and confocal microscopy

Isozyme	Staining relative to control
α	0.98±0.04
βII	1.06±0.19
δ	1.06±0.04
ε	0.95±0.13
ζ	0.95±0.11

The relative levels of PKCα, βII and PKCδ, ε and ζ isozymes, determined as mean integrated fluorescence intensity per unit area, were analysed following activation of v-ABL PTK by temperature switch to 32°C for 6 hours or maintainance at the non-permissive temperature (39°C) for 6 hours. Results are expressed as % of control (maintained at 39°C for 6 hours), with the mean integrated fluorescence intensity determined in each case for a minimum of 20 cells.

The effect of v-ABL PTK activation on the subcellular distribution of PKC isozymes

To investigate further whether PKC plays a role in v-ABL-mediated suppression of apoptosis, the effect of v-ABL activation on PKC isozyme levels and subcellular location was determined. Following immunocytochemical staining of specific PKC isozymes with a panel of isozyme-specific antibodies PKC isozyme distribution was visualised and quantified by laser scanning confocal microscopy. Samples were prepared from cells in which v-ABL was activated by temperature switching from 39°C to 32°C for 6 hours and compared in parallel to cells held at 39°C (i.e. expressing inactive v-ABL). The 6 hour time point was chosen, since that is when increases in DAG, ChoP and IP_3 become apparent (Owen et al., 1993). Moreover, removal of IL-3 from the parental IC2.9 cell line (with no v-ABL) at 32°C results in apoptosis, which is detected 10-22 hours later (Evans et al., 1993). This suggests that the biochemical events critical for the suppression of apoptosis by v-ABL probably occur within a 0-10 hour time period following IL-3 removal. The change in cellular levels of PKC isozymes in IC.DP cells 6 hours after the switch from restrictive to permissive temperatures for v-ABL PTK activity are shown in Table 1. Activation of v-ABL for 6 hours was without significant effect on the overall levels of PKCα, βII, PKCδ, ε or ζ isozyme (P>0.1 Student's t-test). Confocal microscopy confirmed that pretreatment with, and continuous exposure of cells to TPA (166 nM) did not completely downregulate PKCβII and PKCζ 6 hours after the temperature switch (Fig. 3A). Of the two PKC isozymes (PKCβII, PKCζ), which were not completely downregulated by

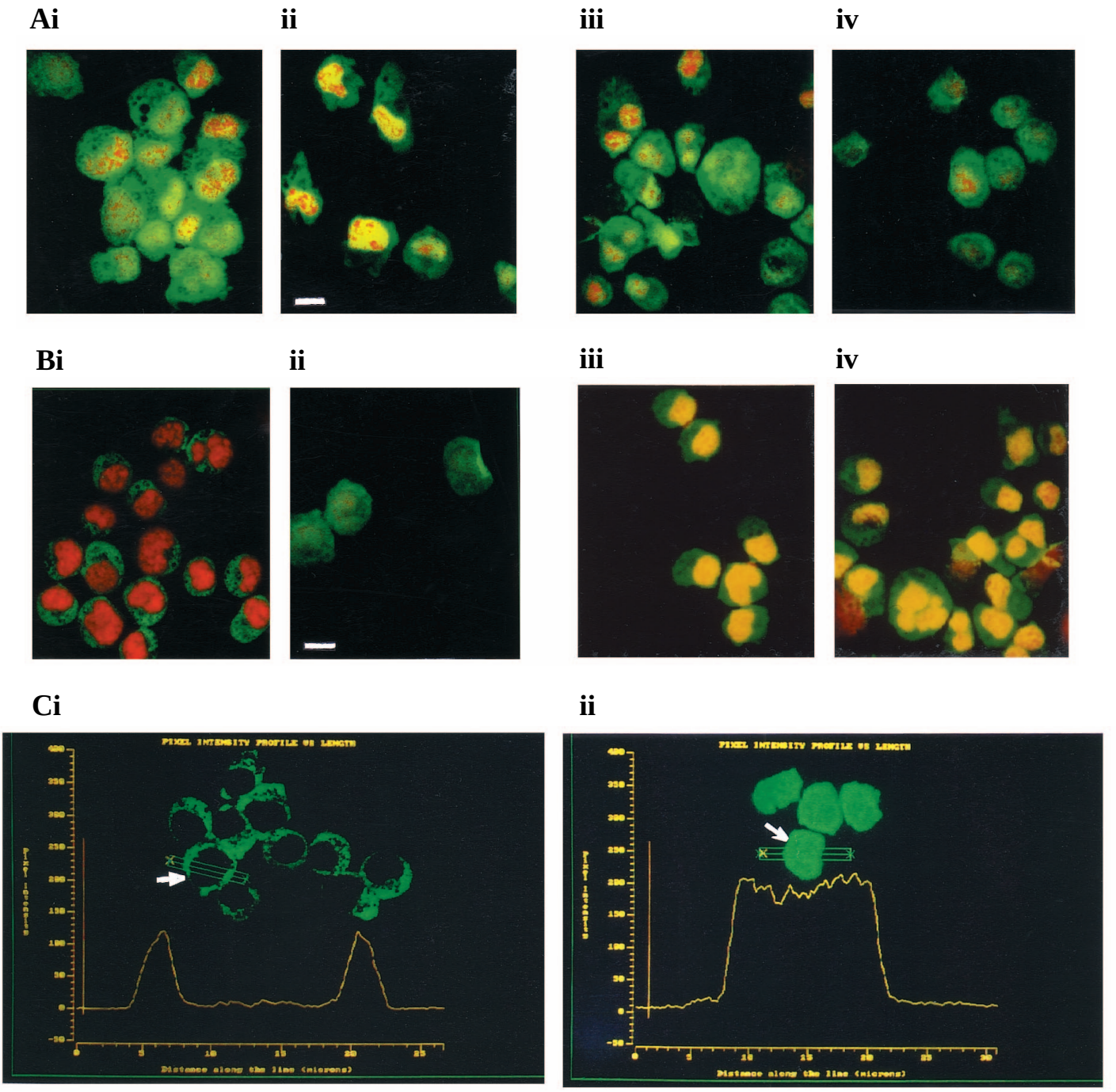

Fig. 3. Immunohistochemical analysis of PKC isozymes in IC.DP cells using laser scanning confocal microscopy. (A) Effect of chronic exposure of TPA (166 nM) on the levels and subcellular localisation of PKCβ_{II} (Ai-ii) and PKCζ (Aiii-iv). Cells were exposed to TPA (166 nM) for 4 hours prior to activation of v-ABL PTK and continuously exposed to this phorbol ester (Aii and Aiv). Control samples were not treated with TPA (Ai and Aiii). Samples were stained with anti-PKCβ_{II} (Ai and Aii) or anti-PKCζ (Aiii and Aiv) specific antibodies with FITC-conjugated second antibody. Nuclei were counterstained with propidium iodide. Yellow fluorescence in these merged images indicates co-localisation of PKC isozyme (FITC, green fluorescence) and DNA (propidium iodide, red fluorescence). Bar, 10 μm. (B) Effect of v-ABL activation on the subcellular localisation of PKCβ_{II} and PKCζ in IC.DP cells. IC.DP cells were held for 6 hours either at the non-permissive (Bi and Biii) or permissive (Bii and Biv) temperature for v-ABL PTK activity. Cell preparations were stained with anti-PKCβ_{II} (Bi and Bii) or anti-PKCζ (Biii and Biv) specific antibodies with FITC-conjugated second antibody. Nuclei were counterstained with propidium iodide. Yellow fluorescence in these merged images indicates co-localisation of PKC isozyme (FITC, green fluorescence) and DNA (propidium iodide, red fluorescence). Bar, 10 μm. (C) Quantification of the nuclear translocation of cPKCβ_{II} 6 hours after activation of v-ABL PTK. Cells were held for 6 hours at the non-permissive temperature (i) or permissive temperature (ii) for v-ABL activity prior to indirect immunostaining with anti-cPKCβ_{II} antibodies. Fluorescence intensity was determined along a linear path, 10 pixels wide, traversing an entire representative cell from each group (indicated by a white arrow). The pixel intensity profile was superimposed upon the fluorescent images: Ci, 39°C (v-ABL inactive); Cii, 32°C (v-ABL active).

Table 2. Effect of v-ABL activation on subcellular location of cPKCβII in IC.DP and IC2.9 cells measured by immunohistochemistry and confocal microscopy

Cell line	Temperature (°C)	% Extranuclear	% Nuclear
IC2.9	39	55±6	45±6
	32	55±5	45±4
ICDP	39	62±2	38±3
	32	54±2	46±2
	(+Calphostin C)	(63±1)	(37±1)

The subcellular location of cPKC βII was assessed in cells following activation of v-ABL PTK by temperature switch to 32°C for 6 hours, or in cells maintained at 39°C (the restrictive temperature). The degree of nuclear staining was determined by assessing the green fluorescence that was coincident with red, propidium iodide, fluorescence. Results are expressed as the % of total cellular staining present in the nuclear and extra-nuclear fractions. Data obtained for IC.DP cells preincubated with calphostin C (150 nM) for 15 minutes prior to temperature switching from 39°C to 32°C for 6 hours are shown in parentheses.

TPA and which are therefore putative survival signal transducers, only the subcellular translocation of PKCβII was effected in response to activation of v-ABL PTK (Fig. 3B). v-ABL activation resulted in an increase in nuclear PKCβII with relatively high levels observed at the nuclear periphery (Fig. 3Bii). Fig. 3Ci and ii both show two groups of cells; the cells on the left-hand side were held at 39°C (v-ABL inactive) whilst the cells on the right-hand side were switched to 32°C (v-ABL active) for 6 hours. Determination of the integrated fluoresence intensity along a 10 pixel wide path traversing a representative cell from each experimental group confirmed the increased nuclear distribution of PKCβII subsequent to v-ABL activation (Fig. 3C). Quantification of the subcellular distribution of PKCβII and PKCζ isozymes underlined that, whilst PKCζ was unaffected, v-ABL stimulated a small but significant increase in nuclear PKCβII (P=0.05, Student's t-test) (Table 2). The observed nuclear translocation is most likely due to the association of PKCβII with the nuclear envelope rather than entry into the nuclear matrix, since the method available for quantification measures changes in PKC levels within the nucleus only. Changes in the nuclear envelope association would therefore not be detected by this method. The actual degree of translocation is therefore likely to be understimated in Table 2. No such change was observed in the IC2.9 parental cell line, indicating that PKCβII nuclear translocation does not simply result from temperature switching. Furthermore, calphostin C was able to inhibit the nuclear translocation of PKCβII, seen subsequent to v-ABL activation (Table 2), concomitant with its inhibition of the suppression of apoptosis by v-ABL.

The effect of activation of v-ABL PTK on PKC activity

In order to confirm that activation of v-ABL stimulates nuclear translocation and to investigate whether there is consequent activation of PKC, IC.DP cells were subjected to subcellular fractionation and in vitro assay of PKC activity. Subcellular fractions were prepared from cells in which v-ABL was activated by temperature switching from 39°C to 32°C for 6 hours and compared with those fractions prepared from cells held at 39°C for 6 hours in parallel. Fig. 4 illustrates the effect of temperature switch upon the subcellular location of PKC assayed by its ability to phosphorylate histone III-S. Whilst the activity of the total cell lysates remained constant following v-ABL PTK activation, there was a 1.97±0.12-fold increase in PKC activity of the nuclear fraction (n=4, ± s.e.m.), which would include the nuclear envelope (Fields et al., 1988). The PKC in the nuclear fraction was activated by PS/DAG and further elevated in the presence of Ca^{2+}, consistent with a cPKC isozyme activity. Since the PKC activity obtained in the total cell lysate remained constant following v-ABL activation, the observed increase in nuclear activity must be due to redistribution of PKC to the nuclear fraction, which is consistent with the observations of PKCβII nuclear translocation shown in Fig. 3B and in Fig. 3C. As PKC translocation is associated with activation, we can conclude that v-ABL activation and suppression of apoptosis is associated with an increase in nuclear PKCβII activity.

DISCUSSION

It has been proposed that all cells are programmed to die by apoptosis unless they receive survival signals (Raff, 1993). Survival of non-leukaemic haemopoietic cells requires the continuous presence of HGFs; removal of such growth factors results in cell death via apoptosis (Williams et al., 1990; Koury, 1992). HGFs exert their effect by binding to specific cell surface receptors to stimulate a range of cellular signalling events. The biochemical events which regulate the suppression

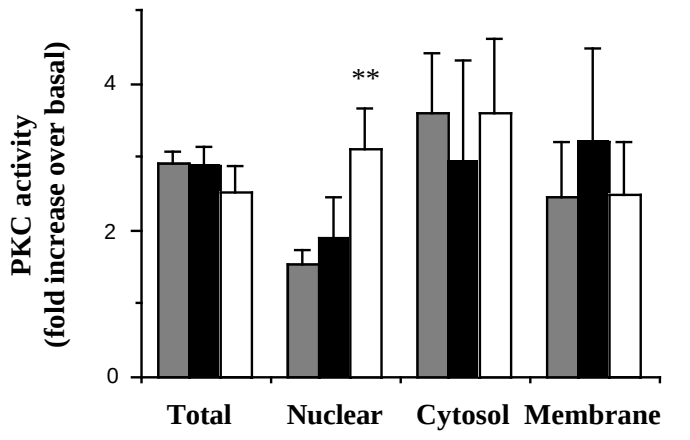

Fig. 4. Effect of v-ABL activation on the subcellular location of PKC activity IC.DP cells. PKC activity was assessed enzymatically in unfractionated cell samples (total PKC activity) together with nuclear, cytosolic and membrane subcellular fractions. Cells were either switched from 39°C to 32°C for 6 hours (v-ABL active, white bars) or held at 39°C (v-ABL inactive, black bars). Control reference samples were assessed at 39°C at time zero (hatched bars) immediately before temperature switch. The data are presented as PKC activity (fold increase over basal activity, i.e. in the absence of PS, DAG or Ca^{2+} for each fraction) Results shown are mean ± s.e.m., n=4 experiments. **, statistically significant from corresponding time 0 control (P<0.02, Student's t-test); all other treatment groups (32°C, 39°C) were not significantly different from their corresponding time 0 controls (P>0.2).

of apoptosis are currently unclear. It is difficult to determine the specific signalling events associated with HGF-stimulated survival of haemopoietic cells, since many HGF act pleiotropically to stimulate proliferation and/or differentiation in addition to permitting cell survival. Several oncogene products promote cell survival via suppression of apoptosis. These include the protein encoded by the *bcl-2* oncogene (Vaux et al., 1988; Nunez et al., 1990) and the v-ABL PTK (Evans et al., 1993). The suppression of apoptosis mediated by v-ABL in IC.DP cells following IL-3 removal occurred in the absence of cell proliferation (Evans et al., 1993), which makes this cell line a useful model system for the study of the cellular signalling events associated with the suppression of apoptosis.

v-ABL is a member of the *src* family of tyrosine kinases, which have a number of defined protein substrates. We have previously shown that v-ABL activation resulted in phospholipid hydrolysis to generate choline phosphate (ChoP) and the second messengers IP_3 and DAG, which is a physiological activator of PKC (Owen et al., 1993). PKC is a family of structurally related serine threonine kinases which differ in their cofactor requirements and substrate specificities. In the present study we provide evidence that v-ABL activation results in an increase in nuclear activity of a Ca^{2+}-dependent cPKC isozyme at a time consistent with events required to suppress apoptosis following IL-3 removal. We have further demonstrated, by immunocytochemical analysis using a panel of isozyme-specific PKC antibodies, that activation of v-ABL PTK leads to specific nuclear translocation of PKCβ_{II}, probably to the perinuclear region. Furthermore, calphostin C inhibited the suppression of apoptosis by v-ABL activation and prevented nuclear translocation and thus activation of PKCβ_{II}. Calphostin C is a relatively selective inhibitor of PKC and was used in preference to other inhibitors, such as staurosporine because of its much reduced activity towards other kinases, specifically protein tyrosine kinases (Kobayashi et al., 1989). There was no evidence that calphostin C affected the increase in tyrosine phosphorylation observed in IC.DP cells subsequent to activation of v-ABL PTK (Owen et al., 1993), as demonstrated by western blotting analysis using an anti-phosphotyrosine antibody (see Results). Furthermore, calphostin C has been shown to prevent both the nuclear translocation of PKCα and macrophage development in bipotential granulocyte-macrophage colony-forming cells (Whetton et al., 1994).

An important question remaining is to determine whether the observed nuclear translocation of PKCβ_{II} subsequent to v-ABL activation is critical to the suppression of apoptosis. Although it has been proposed that the nucleus may not be required for apoptosis (Jacobson et al., 1994), several recent studies suggest that signalling events at the nuclear periphery might be significant for the initiation of apoptosis. For example, translocation of the cyclin A-dependent kinases, Cdc2 and Cdk2, to the nucleus is associated with apoptosis induced by a variety of agents in HeLa cells. These events are inhibited by stable overexpression of the apoptotic suppressor protein BCL-2 (Meikrantz et al., 1994). IGF-1, a fibroblast survival factor has also been demonstrated to stimulate nuclear signalling events, promoting polyphosphoinositide breakdown and nuclear translocation of PKC (Divecha et al., 1991). Interestingly, IGF-1, like v-ABL, does not require de novo protein synthesis for suppression of apoptosis (Harrington et al., 1994).

The maintenance of nuclear integrity associated with the suppression of apoptosis may be regulated by interactions at the nuclear envelope and nuclear matrix, putative recipients of key survival signals. Lamin B, a nuclear envelope protein whose phosphorylation leads to envelope breakdown prior to mitosis and apoptosis, has already been identified as a substrate for PKCβ_{II} in the haemopoietic HL-60 promyelocytic cell line (Fields et al., 1988; Hocevar et al., 1993). Fields and co-workers have recently described a specific PKCβ_{II} activation factor present in the nuclear envelope of HL-60 cells. Association of PKCβ_{II} with this factor is required for its activation (Murray et al., 1994). We have demonstrated translocation of PKCβ_{II} to the perinuclear region after v-ABL activation in non-proliferating cells and conclude that this represents activation of this PKC isozyme. Identification of substrates phosphorylated by PKCβ_{II} following its translocation and activation should clarify its possible role in the suppression of apoptosis by v-ABL. IL-3, the HGF upon which IC2.9 cells are dependent for survival and proliferation, also promotes PKC-mediated phosphorylation of a number of nuclear envelope proteins including lamin (Mays and Kraft, 1990). Thus we can speculate that IL-3 and v-ABL-mediated suppression of apoptosis may involve phosphorylation events elicited by PKC at the nuclear membrane, although the precise molecular basis of their actions remains to be identified.

Interestingly, we have recently shown that a phorbol ester capable of selectively activating PKCβ_I in vitro (Ryves et al., 1991), was able to accelerate the entry of myelomonocytic U937 cells into apoptosis (Pongracz et al., 1993, 1994). Furthermore, despite an initial translocation of this enzyme to the nucleus within 2-10 minutes of treatment with this phorbol ester, by 1 hour, this enzyme was located entirely within the cytoplasm (Pongracz et al., 1994). An interesting speculation, which emerges from these collective data, is that activation and nuclear translocation of PKCβ_{II} may lead to suppression of apoptosis, whilst the activation and cytosolic disposition of the splice variant PKCβ_I may accelerate entry of cells into the apoptotic cell death programme. This possibility requires much further investigation to define the role of PKCβ and its two isoforms in the regulation of apoptosis.

In summary, we have demonstrated that the v-ABL PTK confers a survival advantage on IC.DP cells by the suppression of apoptosis. In this cell type, suppression of apoptosis was associated with perinuclear translocation and activation of a specific PKC isozyme, PKCβ_{II}. The development of leukaemia depends not only on aberrant proliferation and differentiation, but may also reflect an altered rate of leukaemic cell apoptosis. Understanding the signalling events which culminate in the suppression of apoptosis may make it possible to design novel strategies for treatment of leukaemias by revealing specific targets for new anticancer agents.

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REFERENCES

Basu, A., Teicher, B. A. and Lazo, J. S. (1990). Involvement of protein kinase C in phorbol ester-induced sensitization of HeLa cells to cis-diamminedichloroplatinum (II). *J. Biol. Chem.* **265**, 8451-8457.

Bell, R. M. and Burns, D. J. (1991). Lipid activation of protein kinase C. *J. Biol. Chem.* **266**, 4661-4664.

Borner, C., Guadagno, S. N., Fabbro, D. and Weinstein, I. B. (1992). Expression of four protein kinase C isoforms in rat fibroblasts. Distinct subcellular distribution and regulation by calcium and phorbol esters. *J. Biol. Chem.* **267**, 12892-12899.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid dependent protein kinase by tumour promoting phorbol esters. *J. Biol. Chem.* **257**, 7847-7851.

Chapman, R. S., Whetton, A. D. and Dive, C. (1994). The suppression of drug-induced apoptosis by activation of v-ABL tyrosine kinase. *Cancer Res.* **54**, 5131-5137.

Daley, G. Q., Van Etten, R. A. and Baltimore, D. (1990). Induction of chronic myeloid leukaemia in mice by the P210bcr/abl gene of the Philadelphia chromosme. *Science* **247**, 824-830.

Dive, C., Evans, C. A. and Whetton A. D. (1992a). Induction of apoptosis - new targets for cancer chemotherapy. *Semin. Cancer Biol.* **3**, 417-427.

Dive, C., Gregory, C. D., Phipps, D. J., Evans, D. L., Milner, A. E. and Wyllie, A. H. (1992b). Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochem. Biophys. Res. Commun.* **1133**, 275-285.

Divecha, N., Banfic, H. and Irvine, R. F. (1991). The polyphosoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-1) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. *EMBO J.* **10**, 3207-3214.

Elefanty, A. G., Hariharan, I. K. and Cory, S. (1990). bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple neoplasmas in mice. *EMBO J.* **9**, 1069-1078.

Evans, C. A., Owen-Lynch, P. J., Whetton, A. D. and Dive, C. (1993). Activation of the Abelson tyrosine kinase activity is associated with suppression of apoptosis in haemopoietic cells. *Cancer Res.* **53**, 1735-1738.

Farago, A. and Nishizuka, Y. (1990). Protein kinase C in transmembrane signalling. *FEBS Lett.* **268**, 350-354.

Fields, A. P., Pettit, G. R. and May, W. S. (1988). Phosphorylation of lamin B at the nuclear membrane by activated protein kinase C. *J. Biol. Chem.* **263**, 8253-8260.

Fields, A. P., Pincus, S. M., Kraft, A. S. and May, W. S. (1989). Interleukin-3 and bryostatin 1 mediate rapid nuclear envelope protein phosphorylation in growth-factor dependent FDC-P1 hematopoietic cells. A possible role for nuclear protein kinase C. *J. Biol. Chem.* **264**, 21896-21901.

Gerson, S. L. and Trey, J. E. (1988). Modulation of nitrosurea resistance in myeloid leukaemias. *Blood* **71**, 1487-1494.

Gregory, C. D., Dive, C., Henderson, S., Smith. C. A., Wlliams, G. T., Gordon, J. and Rickinson, A. B. (1991). Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* **349**, 612-614.

Harrington, E. A., Bennet, M. R., Fanidi, A. and Evan, G. I. (1994). c-myc induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.* **13**, 3286-3295.

Hincks, J. R., Adlakha, A., Cook, C. A., Johnson, C. S., Furmanski, P. and Gibson, N. W. (1990). In vitro studies on the mechanism of action of hepsulfam in chronic myelogenous leukaemia patients. *Cancer Res.* **50**, 7559-7563.

Hocevar, B. A., Burns, D. J. and Fields, A. P. (1993). Identification of protein kinase C (PKC) phosphorylation sites on human lamin B. Potential role of PKC in nuclear lamin structural dynamics. *J. Biol. Chem.* **268**, 7545-7552.

Hug, H. and Sarre, T. F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291**, 329-343.

Jacobson, M. D., Burne, J. F. and Raff, M. C. (1994). Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* **13**, 1889-1910.

Johnson, G. D., Davidson, R. S., McNamee, K. C., Russell, G., Goodwin, D. and Holborow, E. J. (1982). Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J. Immunol. Meth.* **55**, 231-240.

Karasuyama, H. and Melchers, F. (1988). Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5 using modified cDNA expression vectors. *Eur. J. Immunol.* **18**, 97-104.

Kelliher, M. A., McLaughlin, J., Witte, O. N. and Rosenberg, N. (1990). Induction of a chronic myelogenous leukaemia-like syndrome in mice with v-abl and BCR/ABL (published erratum appears in *Proc. Nat. Acad. Sci. USA* 1990 Nov; 87(22):9072). *Proc. Nat. Acad. Sci. USA* **87**, 6649-6653.

Kobayashi, E., Nakano, H, Morimoto, M and Tamaoki T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159**, 548-553.

Knox, K. A., Johnson, G. D. and Gordon, J. (1993). A study of protein kinase C isozyme distribution in relation to Bcl-2 expression during epithelial cells in vivo. *Exp. Cell Res.* **207**, 68-73

Koury, M. J. (1992). Programmed cell death (apoptosis) in hematopoiesis. *Exp. Haematol.* **20**, 391-394.

McConkey, D. J., Hartzell, P., Jondal, M. and Orrenius, S. (1989). Inhibition of DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. *J. Biol. Chem.* **264**, 13399-13402.

Meikrantz, W., Gisselbrecht, S., Tam, S. W. and Schlegel, R. (1994). Activation of cyclin A-dependent protein kinases during apoptosis. *Proc. Nat. Acad. Sci. USA* **91**, 3754-58.

Murray, N. R., Burns, D. J. and Fields, A. P. (1994). Presence of a βII protein kinase C-selective nuclear membrane activation factor in human leukaemia cells. *J. Biol. Chem.* **269**, 21385-21390.

Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J. P. and Korsmeyer, S. J. (1990). Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived haemopoietic cell line. *J. Immunol.* **144**, 3602-3610.

On, K., Baldwin, S. A. and Whetton, A. D. (1994). Apoptosis is regulated by the rate of glucose transport in an IL-3 dependent cell line. *J. Exp. Med.* **180**, 917-923.

Owen, P. J., Musk, P., Evans, C. A. and Whetton, A. D. (1993). Cellular signalling events elicited by v-abl associated with growth factor independence in an interleukin-3 dependent cell line. *J. Biol. Chem.* **268**, 15696-15703.

Perotti, M., Toddei, F., Mirabelli, F., Valretti, M., Bellomo, G., McConkey, D. J. and Orrenius, S. (1990). Calcium dependent DNA fragmentation in human synovial cells exposed to cold shock. *FEBS Lett.* **259**, 331-334.

Pongracz, J. Burnett, D., Johnson, G. D., Stockley, R. A., Crocker, J. and Lord, J. M. (1993). Molecular events associated with myeloid cell apoptosis, proteins, phosphoproteins and activation of protein kinase C β_I. *J. Pathol.* **170S**, 13.

Pongracz, J., Johnson, G. D., Crocker, J., Burnett, D and Lord, J. M. (1994). The role of protein kinase C in myeloid cell apoptosis. *Biochem. Soc. Trans.* **22**, 593-597.

Raff, M. C. (1992). Social controls on cell survival and death. *Nature* **356**, 397-400.

Rajotte, D., Haddad, P., Haman, A., Cragoe, E. J. Jr. and Hoang, T. (1992). Role of protein kinase C and the Na^+/H^+ antiporter in the suppression of apoptosis by granulocyte-macrophage colony stimulating factor and interleukin-3. *J. Biol. Chem.* **267**, 9980-9987.

Rodriguez-Tarduchy, G. and Lopez-Rivas, A. (1989). Phorbol esters inhibit apoptosis in IL-2 dependent thymocytes. *Biochem. Biophys. Res. Commun.* **164**, 1069-1075.

Roncari, M. and Zeuber, F. (1968). Thermophilic aminopeptidases from Bacillus stearothermophilus 1. Isolation, specificity and general properties of the thermostable aminopepetidase. *Int. J. Protein Res.* **1**, 45-61.

Ryves, W. J., Evans, A. T., Olivier, A. R., Parker, P. J. and Evans, F. J. (1991). Activation of PKC-isotypes alpha, beta I, gamma, delta and epsilon by phorbol esters of different biological activities. *FEBS Lett.* **288**, 5-9.

Stabel, S., Rodriguez-Pena, A., Young, S., Rozengurt, E. and Parker, P. J. (1987). Quantitation of protein kinase C by immunoblot expression in different cell lines and response to phorbol esters. *J. Cell. Physiol.* **130**, 111-117.

Tamaoki, T. (1991). Use and specificity of staurosporine, UCN-01 and calphostin C as protein kinase C inhibitors. *Meth. Enzymol.* **201**, 340-347.

Vaux, D. L., Cory, S. and Adams, J. M. (1988). Bcl-2 gene promotes haemopoietic cell survival and co-operates with c-myc to immortalise pre-B cell lines. *Nature* **335**, 440-442.

Whetton, A. D., Heyworth, C. M., Nicholls, S. E., Evans, C. A., Lord, J. M., Dexter, T. M. and Owen-Lynch, P. J. (1994). Cytokine-mediated protein kinase C activation is a signal for lineage determination in bipotential granulocyte macrophage colony forming cells. *J. Cell Biol.* **125**, 651-659.

Williams, G. T., Smith, C. A., Spooncer, E., Dexter, T. M. and Taylor, D. R. (1990). Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**, 76-79.

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