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Pro-B-Cell-Specific Transcription and Proapoptotic Function of Protein Kinase Cη

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Using a subtractive cloning scheme on cDNA prepared from primary pro-B and pre-B cells, we identified several genes whose products regulate apoptosis. We further characterized one of these genes, encoding protein kinase Cη (PKCη). PKCη transcripts were readily detected in pro-B cells but were absent in pre-B cells. Although both a full-length and a truncated form of PKCη were detectable in bone marrow pro-B cells, transition to the pre-B-cell stage was associated with increased relative levels of truncated PKCη. We found that PKCη is proteolyzed in apoptotic lymphocytes, generating a kinase-active fragment identical to the truncated form which is capable of inducing apoptosis when expressed in a pro-B cell line. Caspase-3 can generate an identical PKCη cleavage product in vitro, and caspase inhibitors prevent the generation of this product during apoptosis in transfected cell lines. Inducible overexpression of either the full-length or truncated form of PKCη results in cell cycle arrest at the G_1/S transition. These results suggest that the expression and proteolytic activation of PKCη play an important role in the regulation of cell division and cell death during early B-cell development.

B-cell development is characterized by the ordered assembly of immunoglobulin (Ig) heavy- and light-chain genes from their component gene segments by a site-specific DNA rearrangement reaction known as V(D)J recombination (61). This reaction is regulated such that heavy-chain genes assemble before light-chain genes, and an individual B cell expresses only one functional gene of each type (allelic exclusion [44, 46]). Heavy-chain protein is expressed on the surfaces of developing pre-B cells along with surrogate light chains and the signal transduction molecules Igα and Igβ in a complex known as the pre-B-cell receptor (pre-BCR). The pre-BCR is a critical regulator of development, responsible for the activation of Ig-light-chain locus rearrangement and the inactivation of allelic heavy-chain locus rearrangement (35, 49, 52). Mutational inactivation of any of the components of the pre-BCR leads to developmental arrest at a distinct stage of B-cell development (13, 23, 24).

Developing pro-B cells which fail to assemble the pre-BCR undergo apoptosis, whereas cells expressing the pre-BCR increase expression of the anti-apoptotic Bcl-x_L gene and survive for an extended period (10). Furthermore, the ongoing expression of surface Ig is essential for B-cell viability (26). Due to the sum of these processes, the great majority of developing B cells fail to survive.

In addition to regulating gene rearrangement and cell survival, the pre-BCR signals specific alterations in the transcription of several developmentally regulated genes, including those encoding Bcl-x, TdT, and λ5, and the germline κ light chain locus (10, 27, 58). In order to more fully define the set of genes regulated by expression of the pre-BCR, we isolated developmentally arrested pro-B cells from RAG1-deficient mice, pre-B cells from RAG1-deficient/μ-transgenic mice (58) and mature B cells from wild-type spleen, and used RNA from these cells to perform representational difference analysis (RDA [19, 29]). This approach led to the isolation of a large set of cDNA fragments whose expression was either positively or negatively regulated by expression of the pre-BCR. Strikingly, many of the genes encode proteins involved in apoptosis. We report here on the regulated expression and posttranslational modification of one of these genes, encoding protein kinase Cη (PKCη), and present evidence suggesting that PKCη may be involved in the regulation of programmed cell death by the pre-BCR.

MATERIALS AND METHODS

Purification of CD19$^+$ B cells. B cells were purified from the bone marrow of RAG1-deficient and RAG1-deficient/μ-transgenic mice (58) and from the spleen of wild-type mice by using biotinylated monoclonal rat anti-mouse CD19 antibody (25) and streptavidin paramagnetic beads (MiniMacs system; Milltenyi Biotech) as previously described (54). In some experiments, less-mature bone marrow B-lineage cells were purified by the depletion of secretory IgM-positive (sIgM$^+$) cells by using a monoclonal rat anti-mouse IgM antibody, yielding a mixed population of pro-B and pre-B cells, followed by selection with biotinylated anti-CD19 antibody. In addition, wild-type pro-B and pre-B cells were processed in a fluorescence-activated cell sorter (FACS) with anti-CD19, -CD43, and -IgM antibodies. The purity of selected populations was assessed by flow cytometry by using biotinylated monoclonal rat anti-mouse CD19, fluorescein isothiocyanate-conjugated monoclonal rat anti-mouse CD43 (17), and phycoerythrin-conjugated goat anti-mouse IgM antiserum (Southern Biotech).

RDA procedure. Cells were pelleted and poly(A)$^+$ RNA was directly purified with the Micro-FastTrack mRNA Isolation Kit (Invitrogen). Poly(A)$^+$ RNA was converted to double-stranded cDNA by using the cDNA Synthesis System (Gibco BRL) according to the manufacturer's instructions. cDNA (2 μg) was then digested with *Dpn*II, phenol extracted, ethanol precipitated, and resuspended in 20 μl of TE (10 mM Tris [pH 8.0], 0.1 mM EDTA). The digested cDNA (12 μl) was then used for RDA as previously described (19). Final difference products after two rounds of subtraction were digested with *Dpn*II and cloned into the *Bam*HI site of pBluescript II KS(+) (Stratagene). Plasmid DNA was obtained by miniprep purification, digested with *Bam*HI, and analyzed on 1.2% agarose gels. Inserts were gel purified, radioactively labelled by random priming (Boehringer Mannheim), and hybridized to Southern blots of amplified cDNA representing pro-B- and pre-B-cell populations. Inserts displaying differential expression were sequenced with an ABI Dideoxy Terminator Cycle sequencing apparatus (Applied Biosystems), and resulting sequences were compared to the GenBank database with the BLAST program (2).

Library screening. The RAG1-deficient/μ-transgenic cDNA library was prepared in λgt22A by using the SUPERSCRIPT lambda system for cDNA synthe-

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sis and λ cloning (Gibco BRL). Plaque hybridizations were performed with replica nitrocellulose filters from plates containing 10^4 cDNA clones. Subtracted RDA probes (described above) were labelled by random priming (Boehringer Mannheim). Hybridizations were carried out for 3 days at 42°C in 25 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 250 mg of denatured salmon sperm DNA per ml, 50% formamide, 10% dextran sulfate, and 2% sodium dodecyl sulfate (SDS). Washings were performed in 2× SSC–0.1% SDS for 45 min at 68°C, followed by autoradiography. Clones hybridizing to subtracted probes were picked and replated for a secondary screen.

Plasmids. Full-length PKCη (FL-PKCη) cDNA (a kind gift from J. F. Mushinski, National Institutes of Health [NIH]) was constitutively expressed in 220-8 cells by using the pEFB expression vector (38) modified by addition of the neomycin resistance gene. FL-PKCη, truncated PKCη (T-PKCη), and mutant T-PKCη (muT-PKCη) were conditionally expressed in 220-8 cells (53) by using a tetracycline-regulated system. 220-8 cell clones containing the pcDNA-tTak regulatory plasmid (57) were provided by Ann Sheehy. FL-PKCη, T-PKCη, and muT-PKCη were cloned into pTet-Splice (57) modified by addition of the neomycin resistance gene. T-PKCη was generated by PCR by using FL-PKCη plasmid DNA as a template, and its integrity was confirmed by DNA sequencing. The primers for this amplification (GCTCTAGAAGCTTGGCAGGGATGGG TCTCC and GGAATTCCTACAGTTGCAATTCCG) included restriction enzyme sites for cloning and were used to amplify 50 ng of plasmid DNA in a 25-cycle PCR at 94°C for 1 min, 66°C for 2 min, followed by a 10-min final extension at 72°C. muT-PKCη was generated by using the Altered Sites II in vitro mutagenesis system (Promega) and was sequenced to confirm mutation. The sequence of the oligonucleotide used to mutate the ATP binding site of PKCη was AGAACTGTACGCCGTGAATTCGCTGAAGAA.

Immunological reagents. Antibodies used in this study were polyclonal rabbit anti-mouse PKCη directed against the unique carboxyl terminus of mouse PKCη (amino acids 669-683) (Santa Cruz Biotechnology) followed by polyclonal donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) and polyclonal anti-human poly(ADP-ribose) polymerase (PARP) from patient sera (a gift of Antony Rosen) followed by polyclonal goat anti-human IgG conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.).

Cell cycle analysis. For cell cycle analysis, cultured cells were pulsed with 10 μM bromodeoxyuridine (BrdU) (Sigma) for 1 h in RPMI-1640 (Mediatech) supplemented with 10% heat-inactivated fetal calf serum, penicillin-streptomycin, and 50 μM β-mercaptoethanol (RPMI–10% fetal calf serum) at 37°C and 5% CO_2. Cells were then fixed in ice-cold 70% ethanol (EtOH) and incubated for 20 min at room temperature, washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (wash buffer), and centrifuged. The pellet was resuspended in 0.5 ml of denaturing solution (2 M HCl, 0.5% BSA), incubated for 20 min at room temperature, centrifuged, and incubated for 2 min at room temperature in 0.5 ml of 0.1 M sodium borate. Cells were washed once in wash buffer and incubated with mouse anti-human BrdU monoclonal antibody (Pharmingen) in wash buffer plus 0.5% Tween 20 for 20 min at room temperature. Cells were then washed once with wash buffer and incubated for 20 min with fluorescein isothiocyanate-conjugated goat anti-mouse IgG at room temperature. After a final wash, cells were resuspended in 7-AAD (a fluorescent dye used to measure DNA content) at 15 mg/ml of PBS. All analyses were performed on a Becton-Dickinson FACScan instrument with CellQuest software.

Gel electrophoresis and immunoblotting. Cells were washed once with PBS and then lysed in SDS sample buffer (5×10^6 cells/80 μl of sample buffer; 10% glycerol, 3% SDS, 50 mM Tris [pH 6.8]). Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce) with BSA as a standard. SDS–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and the product was transferred to nitrocellulose (Schleicher and Schuell) in Tris-glycine-methanol buffer. Membranes were blocked in PBS–0.1% Tween containing 5% nonfat dry milk. Membranes were incubated with a 1/500 dilution of primary antibodies, followed by incubation with a 1/5,000 dilution of donkey anti-rabbit or goat anti-human horseradish peroxidase-conjugated antibodies and detection by enhanced chemiluminescence (Amersham).

Cell culture and induction of apoptosis. 220-8 cells (53) were cultured at 37°C with 5% CO_2 in 10% RPMI medium. Apoptosis was induced by using two different protocols: (i) irradiation for 5 min with UV-B (0.5 mW/cm^2) in PBS followed by incubation at 37°C with 5% CO_2 in 10% RPMI medium for 24 h or (ii) incubation at 37°C with 5% CO_2 in 10% RPMI medium supplemented with 2 μg of etoposide per ml (Sigma) for 48 h. Cell death by apoptosis was confirmed by ethidium bromide visualization of internucleosomal DNA cleavage on 0.7% agarose gels. DNA was purified as previously described (50). 220-8 cells harboring the tetracycline-regulated plasmids were maintained in 10% RPMI medium supplemented with 0.5 mg of G418 per ml, 1.2 μg of mycophenolic acid per ml, 250 μg of xanthine per ml, 15 μg of hypoxanthine per ml, and 1 μg of tetracycline-HCl (Sigma) per ml. For protein induction, cells were washed and cultured in 10% RPMI medium in the absence of tetracycline.

Protease inhibitors. Leupeptin (stock concentration, 4.2 mM in water), pepstatin A (1.4 mM in dimethyl sulfoxide [DMSO]), chymostatin (10 mM in DMSO), and the caspase inhibitors Ac-YVAD-CHO (50 mM in water), Z-VAD-FMK (50 mM in methanol), and Z-DEVD-FMK (50 mM in DMSO) were purchased from Calbiochem. Tosyl-L-lysine chloromethyl ketone (TLCK) (100 mM in water) and tosyl-L-phenylalanine chloromethyl ketone (TPCK) (57 mM in EtOH) were obtained from Boehringer Mannheim. Iodoacetamide (10 mM in water) was obtained from Sigma. Protease inhibitors were added to culture medium immediately after UV-B irradiation.

In vitro translation and caspase cleavage. PKCη was in vitro translated from pBluescript vector by using the TnT T7 Quick System (Promega) according to the manufacturer's instructions. Recombinant caspase-3 with an activity of 250 relative fluorescence units/min/μl when DEVD-AMC was used as a substrate was provided by Christine Kikly (SmithKline). Cleavage reaction mixtures contained 4 μl of in vitro-translated PKCη and 3 μl of purified recombinant caspase-3. Caspase reaction buffer (100 mM HEPES [pH 7.5]–10% sucrose–0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]-10 mM dithiothreitol) was added to bring the total volume of reaction to 30 μl. Reactions were carried out at 37°C overnight (~15 h). Samples were separated by SDS-PAGE and analyzed by Western blot analysis.

In-gel protein kinase assays. In-gel kinase assays were performed as described with minor modification (9, 12). Protein extracts were subjected to electrophoresis on an SDS–4% polyacrylamide stacking gel and an SDS–7.5% polyacrylamide separating gel. Myelin basic protein (MBP) (0.5 mg/ml; Sigma) was added to the separating gel just prior to polymerization. Following electrophoresis, SDS was removed from the gel by washing five times in 50 mM Tris-HCl (pH 7.6) for 15 min each. The gel was subsequently incubated in a solution containing 40 mM Tris-HCl (pH 7.6), 0.1 mM EGTA, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.4 mM dithiothreitol, and 250 μCi of [γ-^{32}P]ATP for 2 h at 25°C. Gels were washed three times with a solution containing 40 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2.5 mM sodium pyrophosphate, and 5% (wt/vol) Dowex 2 × 8 resin (20/50 mesh; Fluka) for 1 h to remove unincorporated ^{32}P. The gel was briefly rinsed with deionized water and fixed with three washes in 10% (wt/vol) trichloroacetic acid at 70°C for 1 h. The gel was then sealed in a plastic bag and exposed directly to film.

RNA PCR analysis. RNA was prepared from cells by a modification of the acid guanidine thiocyanate-phenol-chloroform extraction method (52). RNA PCR assays were performed with randomly primed cDNA (51). Then 2 μl of cDNA was amplified in a 25-μl reaction mixture by using previously described conditions (52). Primers for control H-2 PCR and for the germline kappa transcript were identical to those used previously (52). The PKCη primers were ATGTC GTCCGGCACGATGAA and GACACCGAATATGTACTTC.

RESULTS

Isolation of pro-B-cell- and pre-B-cell-specific cDNA clones. We used the subtractive cDNA cloning strategy of RDA to identify genes whose activity is affected by expression of Ig-heavy-chain protein during B-cell development (19, 29). In wild-type mice, pro-B cells (fractions A to C) are CD43$^+$/CD19$^+$, while pre-B and later-stage B cells are CD43$^-$/CD19$^+$ (fractions D to F [17]). In RAG1-deficient mice, B-cell development is arrested at the CD43$^+$ pro-B-cell stage, while expression of a transgenic heavy-chain protein (μ) in RAG1-deficient/μ-transgenic mice allows progression to the CD43$^-$ pre-B-cell stage (Fig. 1A) (58). Using biotinylated anti-CD19 antibodies and streptavidin paramagnetic beads, we purified CD19$^+$/CD43$^+$ cells from RAG1-deficient mouse bone marrow (Fig. 1A) (pro-B, fractions A to C) and CD19$^+$/CD43$^-$ cells from RAG1-deficient/μ-transgenic mice (pre-B, fraction D). In addition, we purified CD19$^+$ surface IgM$^+$ cells (fractions E and F) from wild-type spleen. cDNA synthesized by using RNA obtained from these various purified cell fractions was used in a series of RDA experiments. We then used subtracted RDA sequences to probe a RAG1-deficient/μ-transgenic pre-B-cell cDNA library.

A total of 95 clones were analyzed: approximately 36 were identified directly by RDA and the remainder were identified by screening the cDNA library with RDA-derived probe populations. Expression patterns of the selected cDNAs were deduced by Northern blot analysis and reverse transcription-PCR (RT-PCR) (data not shown). The genes corresponding to 25 of these cDNA fragments are listed in Table 1. Several of these genes, including those encoding RAG2, the lambda light chain, and Sox4, were previously known to be regulated during B-cell development. Interestingly, several genes revealed by this screen encode proteins with activities related to the redox potential of the cell and to the induction of apoptosis. These

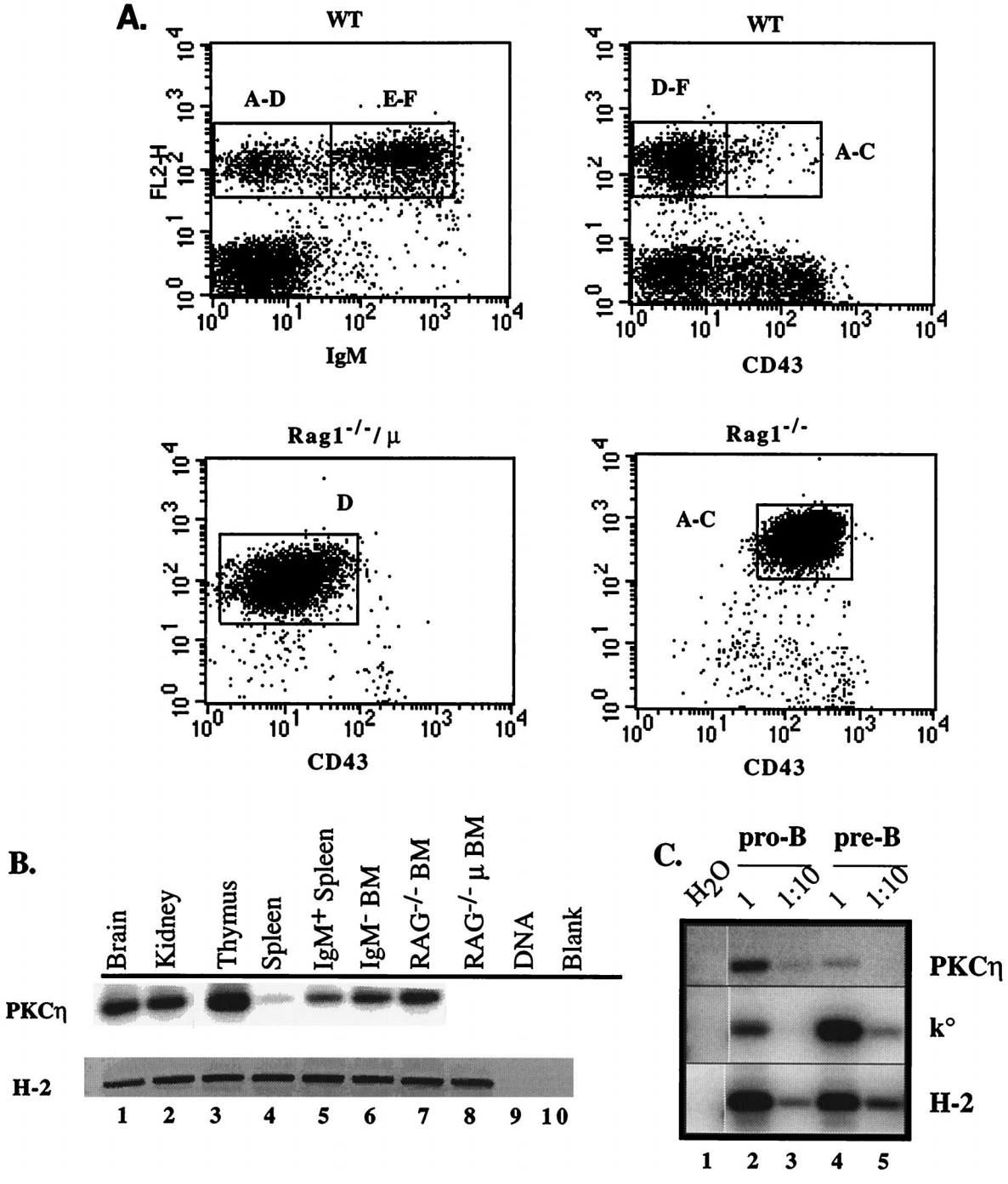

FIG. 1. PKCη expression during B-cell development. (A) Two-color flow cytometric analysis of isolated murine bone marrow cells (upper panels). Unfractionated wild-type bone marrow cells were resolved into fractions A to F by staining with anti-CD19 and either anti-IgM or anti-CD43 monoclonal antibodies (14) (lower panels). $CD19^+$ cells were purified from the bone marrow of RAG1-deficient ($Rag1^{-/-}$) and RAG1-deficient/μ-transgenic ($RAG1^{-/-}/\mu$) mice by using biotinylated anti-CD19 antibody and streptavidin paramagnetic beads. Purified cells were analyzed by staining with anti-CD19 and anti-CD43 antibodies. (B) RT-PCR analysis of PKCη expression. Equivalent amounts of total RNA purified from the indicated organs (lanes 1 through 4), IgM^+ splenocytes (mature B cells, lane 5), $CD19^+$ IgM^- bone marrow (pro-B and pre-B cells, lane 6), RAG1-deficient $CD19^-$ bone marrow (pro-B cells, lane 7; lower panel part A) and RAG1-deficient/μ-transgenic $CD19^+$ bone marrow (pre-B cells, lane 8; lower panels part A) were reverse transcribed and analyzed by PCR with primers specific for PKCη and H-2 (an MHC class I transcript used to confirm the integrity and equivalence of each sample). Controls included PCR analysis of genomic DNA (lane 9) and a mock cDNA reaction (no RNA). Products were separated by gel electrophoresis, blotted to nylon filters, and hybridized with radioactive probes specific for each transcript. Phosphorimages of the blots are shown. (C) RT-PCR analysis of PKCη expression in RNA purified from sorted wild-type pro-B and pre-B cells. Pro-B and pre-B cells were purified by FACS from wild-type bone marrow by using anti-CD19, anti-CD43, and anti-IgM antibodies, and RNA was purified. Aliquots of randomly primed cDNA generated from these RNAs were analyzed for transcription of PKCη, the germline kappa transcript (k^o), and H-2. Both undiluted and 1:10-diluted cDNA was analyzed as indicated. Lane 1 contained H_2O in place of template. The phosphorimage of PCR product blots is shown.

TABLE 1. Subtracted genes[a]

Pre-B-cell-specific genes
Group I
Effector cell protease receptor 1
Stathmin
Calpactin
Topoisomerase II
Inositol triphosphate receptor 1
Ki-67
LPS-inducible gene
Cyclin-dependent kinase
Rag 2
Group II
Mi-2
Y-box transcription factor
ZNF 131
TIS 21
ALL-1
SAM synthetase
p78 kinase
Sox-4
Tax-1
21-kDa polypeptide
Cytochrome *c*
NADH-ubiquinone oxidoreductase
Ubiquitin carboxyl-terminal hydrolase
TAT-binding protein
F1-ATPase
CD33
Lambda light chain
Pro-B-cell-specific genes (group I)
Cathepsin D
TdT
Galectin 9
BCR-1
Lactotransferrin
PKCη

[a] Group-I genes were identified by RDA. Group-II genes were cloned from a pre-B-cell cDNA library probed with RDA-subtracted cDNA. Genes printed in bold have been implicated in apoptosis. Only cDNA showing differential expression patterns are listed. Detailed references for each gene are available upon request.

include proteins located in the mitochondria such as cytochrome *c*, NADH-ubiquinone oxidoreductase, and F1-ATPase. Cytochrome *c* has been shown to induce apoptosis in cell extracts (30), NADH-ubiquinone oxidoreductase is a potent generator of reactive oxygen species (47), and inhibitors of F1-ATPase have been shown to induce apoptosis in the WEHI 231 B cell line (39). Galectin 9 is a recently identified member of a family of proteins which have been shown to stimulate superoxide production (62) and to induce apoptosis of T cells (42, 43). A similar set of genes was also identified in a screen for transcripts induced by p53 expression before the onset of apoptosis (45).

Additional gene products identified by our screen have also been implicated in apoptosis. Increased levels of inositol 1,4,5-triphosphate receptor have been shown to mediate apoptosis in lymphocytes (21). The lysosomal aspartic protease cathespin D has been shown to induce apoptosis in HeLa cells when overexpressed (8) and in PC12 cells following serum deprivation (55). The effector cell protease receptor-1 shares extensive homology with a recently identified gene that functions as an apoptosis inhibitor, survivin (3). Activated Raf kinase, known to regulate cell proliferation and apoptosis, induces the hyperphosphorylation of stathmin and the reorganization of microtubule networks (32). Expression of the antiproliferation gene encoding TIS21 is induced upon DNA damage in cell lines, while cells with a targeted disruption in the TIS21 gene fail to undergo cell cycle arrest in response to DNA damage (48). In addition, TIS21 expression is induced in kidney and liver during acute pancreatitis and is thought to play a role in the control of apoptosis progression in these tissues (11).

Stage-specific expression of PKCη during B-cell development. We chose to further characterize one gene from the group implicated in apoptosis, the gene encoding PKCη. To examine the pattern of expression of PKCη mRNA, we performed RT-PCR analysis on RNA purified from various murine tissues. Amplification of a major histocompatibility complex (MHC) class I gene transcript expressed at similar levels in all cells showed that there were similar amounts of amplifiable cDNA in each sample (Fig. 1B, H-2 lanes). We found high levels of PKCη transcripts in thymocytes, IgM^- bone marrow-derived B-cell precursors from wild-type mice, and bone marrow-derived pro-B cells from RAG1-deficient mice (Fig. 1B, PKCη). Interestingly, we detected extremely low levels of PKCη transcripts in pre-B cells purified from RAG1-deficient/μ-transgenic bone marrow. We confirmed this difference between pro-B and pre-B expression levels by analyzing RNA purified from wild-type $CD19^+/CD43^+$ pro-B and $CD19^+/CD43^-$ IgM^- pre-B cells. We found that pro-B cells expressed approximately 10-fold more PKCη mRNA than pre-B cells (Fig. 1C, compare lanes 2 and 3 with lanes 4 and 5). IgM^+ splenic B-cell RNA contained a higher level of PKCη transcripts than did unfractionated splenocyte RNA, suggesting that mature T lymphocytes express lower levels of PKCη mRNA than do mature B lymphocytes (Fig. 1B, lanes 4 and 5). We also detected expression in cells harvested from brain and kidney (lanes 1 and 2), thus confirming and extending previous reports characterizing the broad pattern of expression of PKCη (4, 36).

PKCη is cleaved during B-cell development. We used antisera specific for PKCη to examine PKCη expression in lymphocytes at the protein level. We detected a single 80-kDa protein, corresponding in molecular mass to FL-PKCη, in thymocytes and splenocytes (Fig. 2A, lanes 1 and 3). However, the pattern of PKCη expression detected by Western blot in RAG1-deficient pro-B cells, RAG1-deficient/μ-transgenic pre-B cells, and a mixture of wild-type pro-B and pre-B cells was more complex. In addition to the 80-kDa band corresponding to FL-PKCη, we also detected a 50-kDa immunoreactive species (Fig. 2B, PKCη lanes 1 to 3). Since our anti-PKCη antiserum was raised against a peptide corresponding to amino acids 669 to 683 in the carboxy terminus of mouse PKCη, we hypothesized that the 50-kDa band contains the C-terminal kinase domain of PKCη. Both the 80- and 50-kDa Western blot bands were specific in that they could be competed by the immunogenic peptide (data not shown).

Because PKC isoforms δ and θ are substrates for proteolysis during apoptosis (7, 9), we asked whether the 50-kDa species observed in developing B cells by Western blot analysis might be due to apoptosis-induced PKCη proteolysis. As an initial test of this idea, we subjected thymocytes and splenocytes to gamma irradiation and performed Western blot analysis of protein lysates. Extracts from irradiated (apoptotic) thymocytes contained a 50-kDa immunoreactive species in addition to 80-kDa FL-PKCη (Fig. 2A, lane 2). This 50-kDa species comigrates with a 50-kDa band detected in lysates of RAG-deficient and wild-type $CD19^+$ bone marrow cells (data not shown). The 50-kDa species is also present at low levels in irradiated primary splenocytes (Fig. 2A, lane 4). Detection of different levels of the 50-kDa fragment in developing T lymphocytes from thymus versus mature splenocytes suggests that

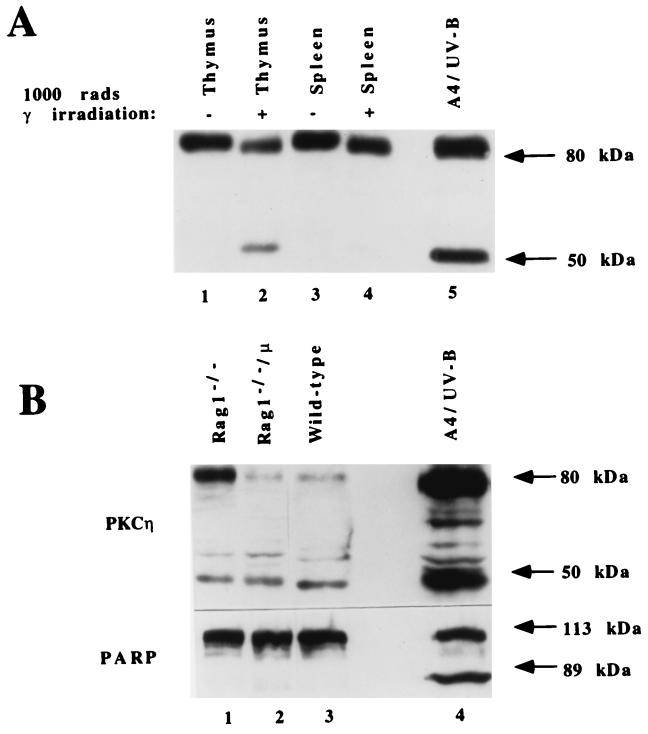

FIG. 2. Western blot analysis of PKCη expression in developing lymphocytes. (A) PKCη expression in gamma-irradiated lymphocytes. Single cell suspensions from thymus and spleen were either cultured directly (lanes labelled −) or subjected to 1,000 rads of gamma irradiation and cultured for 15 h (lanes labelled +). Cells were lysed, and equal amounts of protein (20 μg) were subjected to immunoblot analysis with anti-PKCη antiserum. Lane 5 contains a lysate of a pro-B cell line transfected with an expression vector containing the PKCη cDNA (clone A4) and induced to undergo apoptosis by UV-B irradiation. Arrows indicate FL-PKCη (80 kDa) and a smaller polypeptide which specifically reacts with anti-PKCη antisera (50 kDa). (B) (Upper) PKCη expression in purified B-cell populations. Cells from the bone marrow of RAG1-deficient (pro-B), RAG1-deficient/μ-transgenic (pre-B), and wild-type (pro-B plus pre-B) mice were harvested, and B-cell precursors were purified by positive selection based on the expression of CD19 and negative selection for surface IgM (see Materials and Methods). The lane labelled A4/UV-B shows lysates prepared from transfected pro-B cell clone A4, as described above. Identical cell equivalents (10^6) were analyzed by immunoblotting by using anti-PKCη antisera. Arrows indicate the 80-kDa FL-PKCη and the 50-kDa immunoreactive fragment. (Lower) The immunoblot shown in panel A was stripped and reprobed with anti-PARP antisera. Arrows denote full-length PARP (113 kDa) and cleaved PARP (89 kDa).

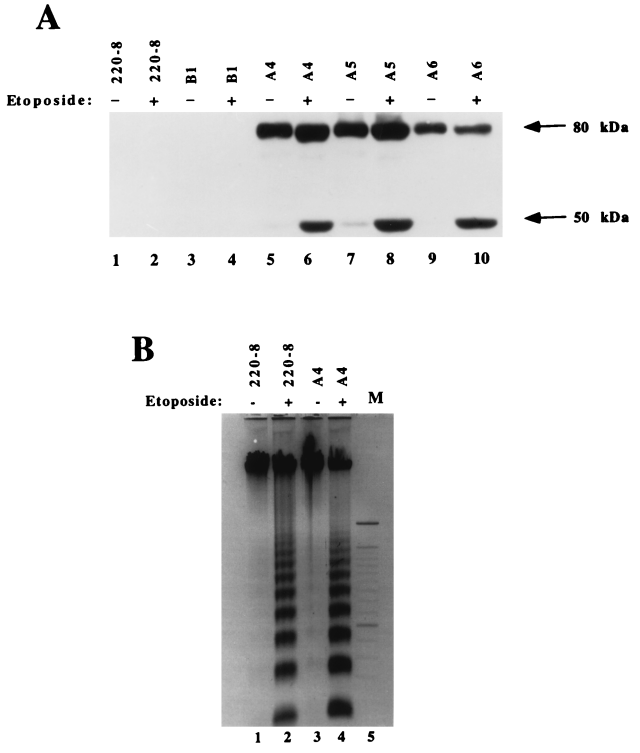

FIG. 3. PKCη is cleaved to a 50-kDa fragment during apoptosis. (A) Western blot analysis of 220-8 pro-B cells transfected with a PKCη expression vector and induced to undergo apoptosis. Untransfected (lanes 1 and 2), empty-vector transfected (vector clone B1, lanes 3 and 4), and PKCη-transfected 220-8 cells (clones A4, A5, and A6, lanes 5 to 10) were cultured in the absence (−) or presence (+) of 2 μg of etoposide per ml for 48 h. Lysates were subjected to immunoblot analysis with anti-PKCη antisera. Arrows indicate FL-PKCη (80 kDa) and cleaved PKCη (50 kDa). Equivalent amounts of protein (20 μg) were analyzed. (B) Etoposide treatment leads to apoptosis, as evidenced by DNA fragmentation. Control untransfected (lanes 1 and 2) and PKCη-expression-vector-transfected 220-8 cells (lanes 3 and 4) were treated with etoposide as described above. DNA was harvested and equal amounts (1 μg) were electrophoresed through 0.7% agarose gels and visualized with ethidium bromide staining.

susceptibility to irradiation-induced apoptosis and proteolysis of PKCη might be developmentally regulated.

The ratio of FL- to cleaved PKCη was much lower in pre-B cells than in pro-B cells (Fig. 2B, compare lanes 1 and 2). Thus, FL-PKCη mRNA and protein are expressed at the pro-B-cell stage, while the cleaved protein remains detectable at the pre-B-cell stage of development, despite greatly diminished levels of transcript. Figure 2B also shows the same protein immunoblot stripped and reprobed with antibodies directed against PARP. PARP is a substrate known to be cleaved during apoptosis in a number of cell culture systems (5, 59). We were unable to detect cleavage of PARP in any stage of B-cell development, although proteolytic cleavage of PARP in PKCη-transfected 220-8 cells undergoing UV-B-induced apoptosis was easily detectable (Fig. 2B, lane 4). These observations led us to hypothesize that PKCη might be involved in apoptosis during lymphocyte development with its cleavage occurring prior to or independent of PARP cleavage.

PKCη cleavage is induced by various proapoptotic stimuli in a transfected pro-B cell line. We used the Abelson virus-transformed pro-B cell line 220-8 to further examine the relationship between PKCη and apoptosis. Endogenous PKCη was undetectable in 220-8 cells by Western blot analysis but was readily detectable after stable transfection with an expression vector containing the FL-PKCη cDNA (Fig. 3A, compare lanes 1, 5, 7, and 9). Treatment of cells with the anticancer drug etoposide, a DNA-damaging agent, results in the induction of apoptosis (5, 63). In 220-8 PKCη transfectants, etoposide-induced apoptosis was associated with cleavage of PKCη to a 50-kDa fragment (Fig. 3A, lanes 6, 8, and 10) and the characteristic internucleosomal cleavage of chromosomal DNA (Fig. 3B, lanes 2 and 4). Similar results were obtained with apoptosis induced by the DNA-damaging agent camptothecin and by UV-B irradiation (Fig. 2A, lane 5; Fig. 2B, lane 4; and data not shown). This 50-kDa apoptosis-induced cleavage product comigrates with the 50-kDa PKCη species expressed in developing B cells (Fig. 2B).

Protease inhibitors block UV-B-induced cleavage of PKCη. Genetic and biochemical studies have demonstrated that proteases of the caspase family are involved in the induction of apoptosis (34, 65). The finding that proteolytic cleavage of PKCδ and PKCθ occurs between aspartic acid and asparagine

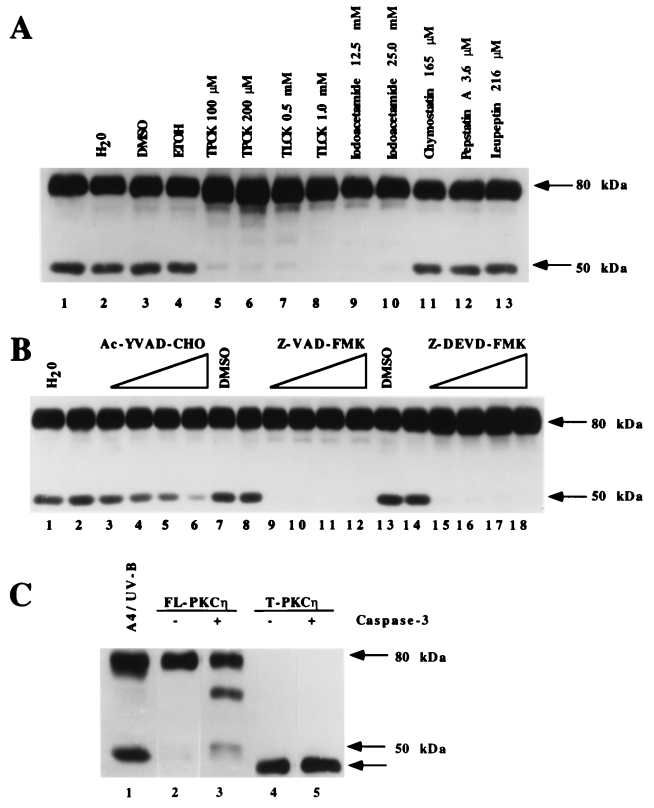

FIG. 4. Effect of protease inhibitors on PKCη cleavage. (A) PKCη-transfected 220-8 cells (clone A4) were irradiated with UV-B and incubated for 24 h in growth medium containing the indicated protease inhibitors. Cells were then lysed, and equivalent amounts of protein (20 μg) were immunoblotted with anti-PKCη antisera. Lysates from a control culture (no protease inhibitors, lane 1) or cultures with the various drug diluents (lanes 2 to 4) are shown. The arrows indicate FL- and cleaved PKCη. (B) Caspase inhibitors block the generation of the 50-kDa PKCη fragment. The caspase inhibitors Ac-YVAD-CHO (lanes 3 to 6), Z-VAD-FMK (lanes 9 to 12), and Z-DEVD-FMK (lanes 15 to 18) were added to cells described in panel A during a 24-h culture period after UV-B treatment, and cells were lysed, and subjected to immunoblot analysis with anti-PKCη antisera. The open triangle indicates increasing levels of inhibitor (25, 50, 100, and 200 μM). Control samples with no protease inhibitor (lanes 2, 8, and 14) or drug diluent (lanes 1, 7, and 13) are shown to the left of each set. The arrows indicate FL- and cleaved PKCη. (C) Recombinant caspase-3 cleaves PKCη in vitro. In vitro-translated FL-PKCη (lanes 2 and 3) or T-PKCη (lanes 4 and 5) was incubated for 15 h at 37°C in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of purified recombinant caspase-3 and subjected to immunoblot analysis with anti-PKCη antisera. Lane 1 contains a lysate of PKCη-transfected cells (clone A4) induced to undergo apoptosis in response to UV-B irradiation. The arrows indicate the intact (80 kDa) and proteolysed (50 kDa) forms of FL-PKCη.

at sites similar to one cleaved in caspase-1 (9, 18) raised the possibility that a caspase family member was involved in cleavage of PKCη. To address this issue, PKCη-transfected 220-8 cells were stimulated to undergo apoptosis by UV-B irradiation in the presence of various protease inhibitors. Chymostatin, pepstatin A, and leupeptin did not prevent cleavage of PKCη (Fig. 4A, lanes 11 to 13). In contrast, TPCK, TLCK, and iodoacetamide abolished the cleavage of PKCη (Fig. 4A, lanes 5 to 10). Thus, the sensitivity of PKCη cleavage to protease inhibitors is identical to that observed for PARP and U1-70-kDa cleavage in apoptotic cells (5, 20).

We next examined the effects of peptide caspase inhibitors on UV-B-induced proteolysis of PKCη in transfected 220-8 cells. While Ac-YVAD-CHO is a specific inhibitor of caspase-1, Z-VAD-FMK and Z-DEVD-FMK are broader in their inhibitory activity, inhibiting caspase-1, caspase-3, and other caspases with varying efficiencies (33). Cleavage of PKCη was insensitive to lower concentrations of Ac-YVAD-CHO but was inhibited at higher concentrations (Fig. 4B, lanes 3 to 6). Z-VAD-FMK and Z-DEVD-FMK, both irreversible inhibitors, completely blocked proteolytic cleavage at all concentrations tested (Fig. 4B, lanes 9 to 12 and 15 to 18, respectively). These findings suggest the involvement of a caspase family member either directly or indirectly in PKCη cleavage.

To further investigate the possibility that PKCη might serve as a caspase substrate, we subjected in vitro transcripts of the PKCη cDNA to in vitro translation and digested the reaction products with purified recombinant caspase-3. In addition, we generated a mutant PKCη cDNA which utilizes an internal ATG initiation codon present in the third variable region (V3) of PKCη (see Fig. 6A). This construct was designed to generate a C-terminal PKCη fragment of a size similar to that of the apoptosis-associated cleavage product. This T-PKCη cDNA was also transcribed and translated in vitro and then subjected to recombinant caspase-3 cleavage. T-PKCη was several kilodaltons smaller than the in vivo cleavage product of FL-PKCη, suggesting that the cleavage site in vivo is closer to the N terminus than the artificial start site used in the T-PKCη constructs (Fig. 4, compare lanes 1 and 4). Treatment of FL-PKCη with caspase-3 produced a 50-kDa proteolytic fragment which comigrated with the in vivo apoptosis-associated fragment of PKCη (Fig. 4C, lanes 1 and 3). A second in vitro cleavage product was also detected, but this fragment was never detected in vivo. In contrast, T-PKCη was not susceptible to recombinant caspase-3 digestion in vitro (Fig. 4C, lane 5). Thus, PKCη is a substrate for caspase-3 in vitro, cleaving a site in or upstream of the V3 region.

Cleavage of PKCη is associated with activation of its kinase function. To explore the biological significance of PKCη cleavage in developing B cells, we assessed whether cleavage of PKCη during apoptosis resulted in activation of its kinase function. An in-gel kinase assay was used to detect phosphorylation of a known substrate for all PKC isoforms, myelin basic protein (9). Kinase activity was restricted to PKCη-transfected cells induced to undergo UV-B-induced apoptosis (Fig. 5B, lane 6) and comigrated with the 50-kDa fragment detected by immunoblot analysis in apoptotic cell lysates (Fig. 5A, lane 2). As predicted, the FL-PKCη protein did not have kinase activity in the absence of added coactivators. Taken together, these findings indicate that cleavage of PKCη during apoptosis in 220-8 cells is associated with activation of its kinase function.

Inducible expression of either FL-PKCη or T-PKCη in transformed pro-B cells alters cell cycle progression. To determine whether PKCη contributes to apoptosis, we expressed FL-PKCη, T-PKCη, and a kinase-deficient muT-PKCη (Lys-383 in the ATP-binding site mutated to Asn) in 220-8 cells by using a tetracycline-regulated expression system (57). A diagram of the FL-PKCη, T-PKCη, and muT-PKCη constructs is shown in Fig. 6A. These constructs were expressed under the control of the tTA transactivator, comprised of the *Escherichia coli* tetracycline repressor fused to the transactivation domain of the herpes virus VP16 protein. The addition of tetracycline to the culture medium blocks transcription of PKCη by preventing binding of tTA to *tetO* operator sequences present within the promoter of the PKCη expression construct. We used Western blot analysis to confirm the expression of the various PKCη constructs upon tetracycline withdrawal (Fig. 6B). As described above, T-PKCη and muT-PKCη migrated with slightly faster mobility on an SDS-polyacrylamide gel than the UV-B-induced 50-kDa cleavage product of FL-PKCη.

The induction of FL- and T-PKCη protein expression led to

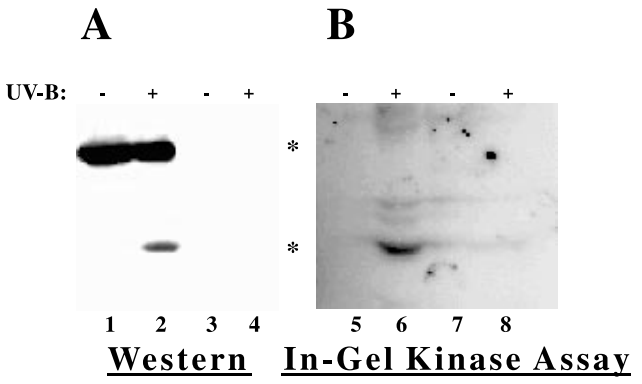

FIG. 5. Apoptosis activates a 50-kDa kinase in PKCη-transfected 220-8 cells. (A) Western blot analysis of PKCη-transfected 220-8 cells induced to undergo apoptosis by UV-B treatment. PKCη-transfected (clone A4; lanes 1 and 2) and untransfected (lanes 3 and 4) 220-8 cells were treated with UV-B, cultured for 24 h, and lysed. Equal amounts (20 μg) of lysate were subjected to immunoblot analysis with anti-PKCη antisera. Asterisks denote the 80-kDa and 50-kDa FL- and cleaved PKCη proteins, respectively. (B) In-gel kinase assay. Lysates (20 μg) from cells described in panel A were electrophoresed through gels containing 0.5 μg of myelin basic protein per ml incorporated in the gel matrix. Following gel electrophoresis, SDS was washed from the gels, and a kinase reaction with [γ-^{32}P]ATP was carried out in vitro. After unincorporated radioactive material was removed, the gel was fixed and exposed to film. A phosphorimage of the gel is shown.

marked decreases in the proliferation of 220-8 cells as compared to empty vector control and muT-PKCη transfectants. The doubling time of control cells was, on average, 12 h, whereas cells expressing FL-PKCη or T-PKCη doubled every 18 and 20 h, respectively (data not shown). MuT-PKCη expression had no effect on the growth rate of transfected cells. Previous studies showing that NIH 3T3 fibroblasts expressing FL-PKCη exhibit diminished growth rates are consistent with these observations (31). To further characterize this effect, we labelled cells in culture with the thymidine analog BrdU and used flow cytometry to analyze cell cycle status. BrdU-pulsed cells were harvested, permeabilized, and stained with anti-BrdU antibody and 7-AAD. FACS analysis of control cells showed a normal pattern of cell cycle distribution (Fig. 7A). However, induction of expression of either FL- or T-PKCη decreased the number of cells that progressed from the G_0/G_1 phase into the S and G_2/M phases of the cell cycle (Fig. 7A and B). Expression of muT-PKCη had little effect on cell growth. Taken together, these results indicate that expression of either FL- or T-PKCη causes a block in cell cycle progression at the G_1/S transition and that this activity requires its kinase function. Moreover, these data suggest a possible role for PKCη in cell cycle regulation in developing B cells.

Induction of apoptosis by overexpression of PKCη catalytic fragments. Having found that PKCη was cleaved into a kinase-active form during apoptosis, we sought to clarify the role of the catalytic fragment of PKCη in contributing to apoptosis itself. We assayed the transfectants described above for endonucleolytic cleavage of nuclear DNA by agarose gel electrophoresis (Fig. 7C). Induction of T-PKCη expression was associated with DNA fragmentation indicative of apoptosis (lanes 10 and 12). This was not observed upon induction of FL-PKCη (lanes 5 to 8) or control-vector transfectants (lanes 1 to 4). Furthermore, the kinase-inactive muT-PKCη failed to induce DNA fragmentation (lanes 13 to 16), supporting a role for the C-terminal kinase domain of PKCη in the apoptotic pathway.

DISCUSSION

Members of the PKC family are serine and threonine kinases with a wide range of physiological functions. These en-

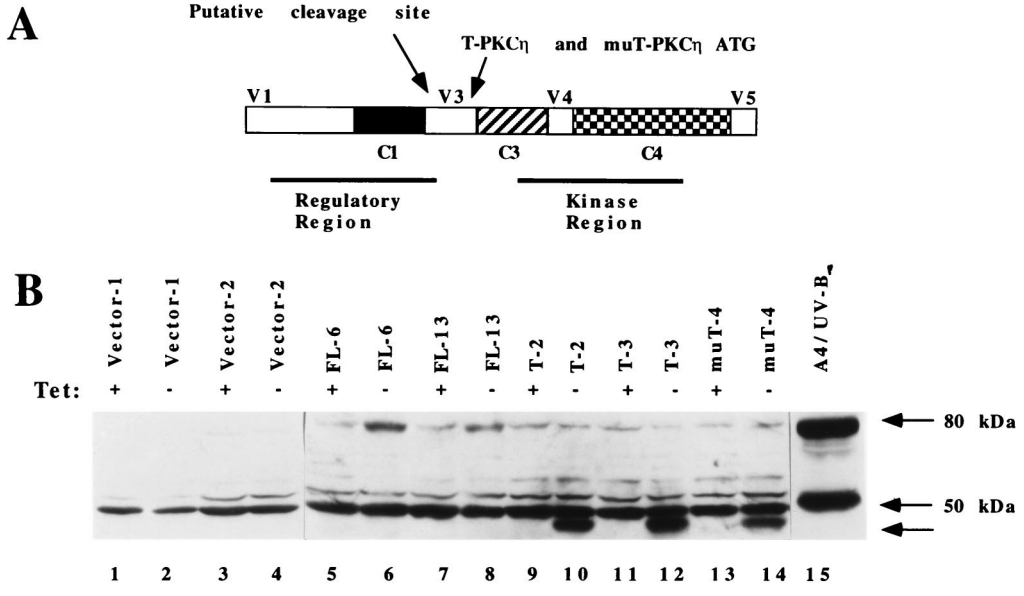

FIG. 6. Tetracycline-regulated expression of PKCη in transfected cell lines. (A) Schematic of PKCη. The FL-PKCη construct contains both the regulatory regions (V1 and C1) and the kinase regions (C3, V4, C4, and V5) separated by V3. The truncated constructs (T-PKCη and muT-PKCη) contain only the kinase region and use internal ATGs present in the V3 region to initiate translation. MuT-PKCη contains Asn substituted for Lys403 in the ATP binding site. The putative site for apoptosis-induced cleavage is shown upstream of the ATG in V3. (B) Western blot analysis of tetracycline-regulated expression of PKCη. Extracts (20 μg) from vector-transfected (vector clones 1 and 2; lanes 1 to 4), FL-PKCη-transfected (clones 6 and 13; lanes 5 to 8), T-PKCη-transfected (clones 2 and 3; lanes 9 to 12), and muT-PKCη-transfected (clones 4 and 8; lanes 13 and 14) 220-8 cells grown for 96 h in the presence (+) or absence (−) of 1 μg of tetracycline per ml were subjected to immunoblot analysis by using anti-PKCη antisera. The last lane, labelled A4/UV-B, shows PKCη-transfected clone A4 induced to undergo apoptosis by UV-B treatment. Arrows denote 80-kDa FL-PKCη, 50-kDa cleaved PKCη, and a slightly smaller T-PKCη fragment.

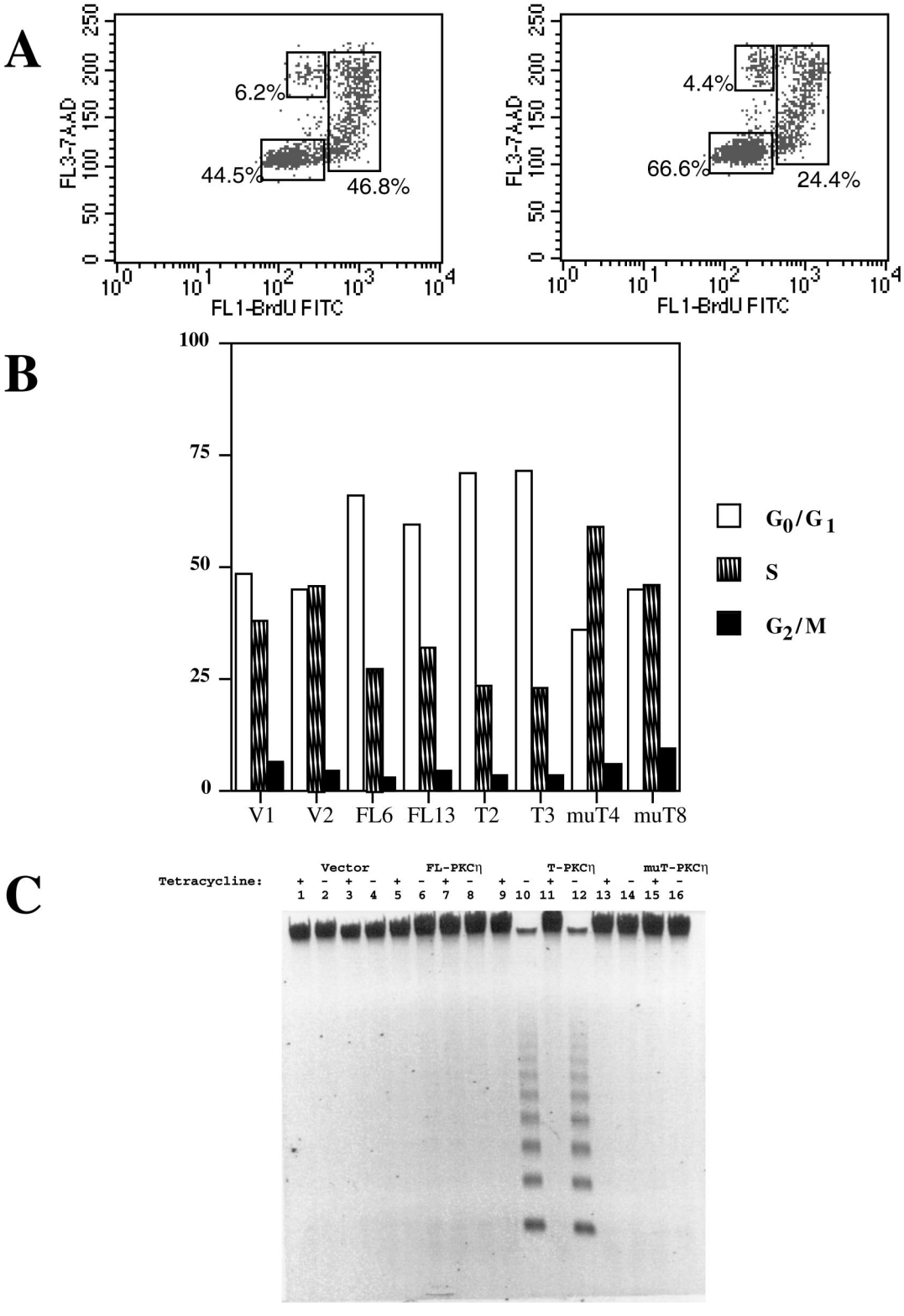

FIG. 7. T-PKCη expression arrests cell cycle progression in G_1 and induces apoptosis. (A) Flow cytometric analysis of cell cycle status. Cells (as described in the legend to Fig. 6B) were grown in the absence of tetracycline for 96 h and pulsed with 10 μM BrdU for 1 h. Cells were then permeabilized, stained with monoclonal anti-BrdU antibody and 7-AAD, and analyzed for DNA content by flow cytometry. Representative analyses of empty-vector transfected control cells (clone V2) (left) and T-PKCη-transfected cells (clone T2) (right) are shown with the boxed regions indicating cells in G_0/G_1, S, and G_2/M. Each cell culture was subjected to identical analysis. (B) The percentage of cells in each phase of the cell cycle is depicted in a bar graph. The identity of each clone is indicated below each set of bars. Flow cytometry data was gated on live cells by using forward and side-scatter criteria. (C) Cells (as described in the legend to Fig. 6B) were grown for 96 h in the presence (+) or absence (−) of 1 μg of tetracycline per ml, and DNA fragmentation was monitored by gel electrophoresis in 0.7% agarose gels. A digital photograph of the ethidium-stained gel is shown.

zymes are activated upon external stimulation of cells by various ligands, including hormones, neurotransmitters, and growth factors (reviewed in reference 40). The 11 known isoforms of PKC have been divided into the classical (cPKC; α, β, and γ), novel (nPKC; δ, ε, η, θ, and μ), and atypical (aPKC; ζ and λ) groups (1). The Ca^{2+}-dependent cPKCs contain the conserved regulatory regions C1 and C2, while the Ca^{2+}-independent nPKC and aPKC isoforms lack the Ca^{2+}-binding C2 domain (40).

Proteolytic cleavage is known to regulate the activity of several PKC isoforms. cPKCs undergo cleavage in V3 by the calcium-activated proteases calpain I and II, deleting the C1 and C2 regulatory regions and resulting in kinase-active fragments (22). Other studies have shown that the PKCδ and θ isoforms are cleaved and activated by the cysteine protease caspase-3 in cells induced to undergo apoptosis (7, 9). Caspase-3-mediated cleavage of PKCδ at a DMQD/N site, and PKCθ at a DEVD/K site, deletes the C1 regulatory domain. Overexpression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L blocked cleavage of both kinases, while overexpression of the cleaved, kinase-active PKCθ fragment resulted in apoptosis (7, 9).

We found that PKCη mRNA is expressed at high levels in purified pro-B cells and early-stage thymocytes, and at lower levels in purified pre-B cells, mature B cells, and unfractionated spleen (Fig. 1). Analysis of PKCη at the protein level revealed a more complex pattern of expression (Fig. 2). FL-PKCη was predominant in purified bone marrow pro-B cells and nearly absent from pre-B cells. The truncated form of PKCη was present in similar amounts at both developmental stages, but the ratio of cleaved to full-length protein was clearly increased in pre-B cells.

Thymocytes and unfractionated bone marrow cells cleave endogenous PKCη into a 50-kDa fragment in response to gamma irradiation (Fig. 2A and data not shown). While not expressed in a variety of transformed pro-B cell lines, PKCη is cleaved in a pro-B cell line transfected with PKCη cDNA and induced to undergo apoptosis with a variety of DNA-damaging agents. Surprisingly, PKCη was relatively resistant to irradiation-induced cleavage in mature splenic lymphocytes, suggesting that susceptibility of PKCη to irradiation-induced apoptosis and proteolysis may be a developmentally regulated characteristic of B and T lymphocytes.

Inducible expression of either FL- or T-PKCη in 220-8 cells led to a cell cycle arrest (Fig. 7B). In the case of the truncated protein, this arrest was accompanied by apoptotic cell death (Fig. 7C). Both of these effects required intact kinase activity, as the kinase-inactive mutant failed to induce cell cycle arrest or apoptosis. Previous reports have shown that ectopic expression of FL-PKCη in NIH 3T3 fibroblasts blocked phosphorylation of Rb protein and inhibited cell growth in quiescent cultures restimulated to enter the cell cycle (31). PKCη inhibition of cell growth in these systems correlated with increased expression of cyclin E and of the cyclin-dependent kinase inhibitors p21 and p27. Furthermore, in contrast to control NIH 3T3 cells, cells transfected with PKCη could be induced to undergo adipocyte differentiation. In the epidermis, high levels of PKCη expression are detected in the suprabasal layers where keratinocytes undergo differentiation (41). These data are consistent with a model where altered expression of cell cycle-related genes may contribute to the ability of PKCη to promote cellular differentiation. Thus, PKCη can regulate the cell cycle, promote differentiation, and contribute to apoptosis. The existence of two forms of PKCη, having overlapping and distinct effects, complicates analysis of the potential roles of PKCη in B-cell development, however.

How is the truncated form of PKCη generated in developing B cells? It is possible that T-PKCη is generated by the initiation of translation at an internal ATG within its mRNA. Examination of the nucleotide sequence of PKCη revealed an ATG codon which might generate a protein of the approximately correct size. We do not think this is the case, however, for the following reasons. First, in vitro translation of FL-PKCη mRNA did not lead to the generation of a 50-kDa protein (Fig. 4C). Second, our engineered truncated protein uses the ATG in question and generates a fragment significantly smaller than the T-PKCη generated by the induction of apoptosis in cells expressing the full length protein (Fig. 4C and 6B). Finally, at the pre-B-cell stage in B-cell development where we detect the greatest fraction of cleaved PKCη, we fail to detect any PKCη mRNA (Fig. 1B and 2B).

The correlated appearance of the 50-kDa form of PKCη with the induction of apoptosis suggests that it may be the proteolytic target of a caspase. In fact, our inhibitor studies show that inhibitors which specifically block caspase-3 prevent the generation of the 50-kDa PKCη fragment (Fig. 4A and B). Also, FL-PKCη is cleaved by purified recombinant caspase-3 in vitro (Fig. 4C). In this regard it is worth noting that although PKCη lacks the preferred caspase-3 cleavage site DEXD, a potential caspase-3 site (NKVD) occurs at amino acids 228 to 231 (4). Aside from a stringent requirement for Asp in P_1, caspase-3 can accommodate other amino acids in $P_{2\text{-}4}$ (60). Hence, both in vivo and in vitro studies point to caspase-3 as the protease which cleaves PKCη in developing B cells. We are currently attempting to determine the in vivo cleavage site by direct peptide sequence analysis. Finally, it remains possible that caspase-3 activates a different protease that is directly responsible for PKCη proteolysis.

Does PKCη proteolysis regulate apoptosis of developing B cells? Death is a frequent outcome for cells at each stage of B-cell development. Pro-B cells that fail to generate an in-frame heavy-chain-gene rearrangement die by apoptosis. Forced expression of the anti-apoptotic gene encoding Bcl-x_L rescues these cells but does not promote their further development (10). Pre-B cells which fail to generate an in-frame light-chain-gene rearrangement also fail to survive. This is most obvious in RAG1-deficient/μ-transgenic mice where mutant pre-B cells are continuously generated and cannot mature but achieve steady-state numbers nonetheless (~10 to 20% of nonerythroid marrow) (56, 58). Immature B cells expressing autoreactive surface IgM either successfully edit their receptors or undergo apoptotic cell death (reviewed in reference 14). Finally, it was shown recently that continuous expression of surface Ig is required for survival of mature peripheral B cells (26). Hence, B cells at every stage in their development are poised to undergo apoptosis.

During apoptosis, specific cellular proteins including PARP, U1-70 kDa, and lamin A are targeted for proteolysis by caspases (reviewed in reference 6). The consequences of these proteolytic events, in particular whether the proteolytic fragments themselves play a role in the execution pathway, in many instances remain uncertain. The results reported in this paper may shed some light on this question.

A decline in PKCδ, -ε, and -θ levels and generation of proteolytic fragments were reported to occur during later stages of Fas-mediated apoptosis (37), suggesting that the generation of these fragments and reduced expression occurs after commitment to the execution stage of apoptosis. The diminished levels of PKCη mRNA and increased fraction of cleaved PKCη protein we observed in RAG1-deficient/μ-transgenic and wild-type pre-B cells suggest that PKCη might be playing a role in the apoptosis of pre-B cells in vivo. Surprisingly, we failed to

detect PARP cleavage at this stage in development. PARP is a known substrate for cleavage during Fas-mediated apoptosis (16) and is cleaved in PKCη-transfected 220-8 cells undergoing UV-B-induced apoptosis (Fig. 2B). Our results suggest that PKCη cleavage might occur prior to PARP cleavage during apoptosis in B cells. Alternatively, cleavage of PKCη may occur independently of PARP cleavage during an alternative pathway of apoptosis. This PARP-independent apoptosis pathway may be limited to a specific stage of B-cell development. It is also possible that PKCη cleavage in bone marrow pre-B cells is a developmentally regulated event unrelated to apoptosis. If this were the case, the cleaved form of PKCη might serve some other role in these cells.

We detected the greatest proportion of cleaved PKCη in RAG1-deficient/μ-transgenic pre-B cells. This led us to consider at which corresponding stage of wild-type B cell development PKCη might be involved. RAG1-deficient pro-B cells cannot assemble the pre-BCR due to their absolute block in gene rearrangement. These pro-B cells must be dying, since they are continuously generated and do not escape to the periphery, but their overall numbers within the marrow do not inexorably increase (58, 64). Wild-type pro-B cells that fail to productively rearrange a heavy-chain gene undergo apoptosis which can be partially blocked by *bcl-x* (10). Detection of some PKCη cleavage product in RAG1-deficient pro-B cells leads us to suggest that PKCη cleavage may be involved in this type of pro-B-cell apoptosis.

Upon initial expression of the pre-BCR, developing B cells undergo a period of proliferative expansion during which gene rearrangement is suspended (15). This is followed by a period of quiescence and active V(D)J recombination during what is called the small, resting pre-B-cell stage (17, 28, 35). Pre-BCR expression results in the transcriptional inactivation of λ5 expression and the subsequent loss of the pre-BCR (28, 58). We propose that unless the surrogate light chains are replaced by a true light chain (the product of successful κ or λ gene rearrangement), these pre-B cells are destined to undergo apoptosis. We believe that it is this apoptotic event which predominates in the RAG1-deficient/μ-transgenic pre-B-cell population since, due to their RAG-deficiency, these cells are unable to generate a functional light-chain gene. PKCη cleavage may be involved in an apoptosis pathway which deletes wild-type cells unable to generate a functional light chain. Genetic experiments in which PKCη expression is disrupted in developing B cells should shed more light on its precise role in the development of this lineage.

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