

A Chromatin Immunoprecipitation Screen Reveals Protein Kinase C β as a Direct RUNX1 Target Gene*

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RUNX1 (also known as AML1) is a DNA-binding transcription factor that functions as a tumor suppressor and developmental determinant in hematopoietic cells. Target promoters have been identified primarily through the use of differential expression strategies and candidate gene approaches but not biochemical screens. Using a chromatin immunoprecipitation screen, we identified protein kinase C β as a direct RUNX1 target gene and demonstrate that endogenous RUNX1 binds the chromatinized protein kinase C β promoter of U937 cells. A phylogenetically conserved RUNX1-binding site within the PKC β promoter binds RUNX1 in electrophoretic mobility shift analyses and confers RUNX1 responsiveness on a heterologous promoter. Changes in RUNX1 activity affect endogenous protein kinase C β expression, and a dominant-negative form of RUNX1 protects U937 cells from apoptotic stimuli previously shown to be dependent on protein kinase C β . This protection can be reversed by the ectopic expression of protein kinase C β . Together these findings demonstrate that protein kinase C β is a direct, downstream target of RUNX1 and links RUNX1 to a myeloid apoptotic pathway.

Three RUNX transcription factors make up the mammalian family named for homology with the *Drosophila* pair-rule gene, *RUNT* (1). The RUNX proteins are DNA-binding transcription factors with the context-specific capability of activating or repressing gene expression (1, 2). The transcription factors are important developmental determinants with oncogenic potential (1). Loss of function results in malignancy, in the cases of *RUNX1* and *RUNX3*, and dysplasia in the case of *RUNX2* (3–7). Gain of function also appears to have oncogenic effects, because aberrant activation of any of the *RUNX* genes contributes to dysregulated proliferation (8–12). Thus, the transcription factor family has complex roles in growth and development, and either loss or gain of activity can predispose a cell to maturation defects or transformation.

Identification of RUNX target genes is essential for understanding the cellular effects of the transcription factors. RUNX1 has been the most extensively analyzed family mem-

ber, and numerous target pathways have been identified (13–15). Most RUNX1 target genes have been revealed by identifying consensus motifs within candidate target promoters or by performing differential expression screens (13, 15). These approaches have been invaluable for identifying RUNX1 target pathways. However, candidate gene approaches may overlook targets with one or more base deviations from the perfect TG(T/C)GGT consensus or uncharacterized regulatory elements distant from promoters regions (16, 17). Differential expression strategies likely omit target genes that undergo small changes in expression or genes that change only under select culture conditions. It is, therefore, likely that many RUNX1 target genes important for cellular function remain undefined.

Chromatin immunoprecipitation (ChIP)¹ is a revolutionary biochemical technique for demonstrating direct interactions between transcription factors and their target promoters (18). The assay allows for the biochemical enrichment of chromatin fragments associated with endogenous factors and is commonly used for candidate target gene validation (18). Recently, ChIP was shown to be a potent method for identifying novel target promoters (19–22). Immunoprecipitated chromatin fragments were cloned and sequenced to reveal transcription factor targets (19). The unbiased ChIP screen is distinct from other target identification strategies because it does not rely on changes in gene expression or the disproportionate presence of transcription factor consensus sites within known regulatory regions. The assay, thus, promises to identify a unique subset of target genes that would be missed by conventional strategies. Additionally, because ChIP exclusively identifies direct biochemical targets, the screen is insulated from the indirect and often confusing cellular effects that result from alterations of transcriptional pathways.

Using a RUNX1 ChIP screen, we identified protein kinase (PKC β) as a direct RUNX1 target. Changes in RUNX1 activity affect PKC β expression and a well characterized, PKC β -dependent apoptotic pathway of myeloid cells. Thus, RUNX1 regulation of PKC β is an important aspect of normal and pathological biology of myeloid cells.

EXPERIMENTAL PROCEDURES

Cell Culture—U937 cells were grown in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine (Invitrogen), and 100 units/ml penicillin-streptomycin (Invitrogen). Transductions were performed by spinoculation (centrifugal inoculation) as described previously (23, 24). Cell lines transduced with LXIN-based retroviral vectors were maintained on media containing 1

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¹ The abbreviations used are: ChIP, chromatin immunoprecipitation; PKC β , protein kinase C β ; DN, dominant-negative; AML, acute myeloid leukemia; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift analysis.

mg/ml geneticin (Invitrogen). Cells transduced with MigR1-based retroviral vectors were sorted to purity using a FACS Vantage cell sorter (BD Biosciences) 24 h after transduction.

Constructs—A sequence-encoding FLAG sequence was added to the 5' end of *RUNX* genes by PCR. *RUNX1*, *RUNX1* DN (*i.e.* *AML1a* (25)), and *PKC β* (ATCC) were cloned into LXIN (Clontech) or MigR1 (24) bicistronic proviral vectors. The FLAG-*RUNX* DNA-binding domain construct encodes the first 177 amino acids of RUNX1 and was cloned into LXIN. The *PKC β* promoter construct encompassing bases -17 to -1003, with respect to the translational start site, was cloned into PGL2-basic vector (Promega, Madison, WI) using PCR. Complementary oligomers containing tandem repeats of the bases -562 to -532 were annealed and cloned into the PGL2-promoter vector (Promega). Mutant oligomers contain the sequence GCTAGC in place of the RUNX1 consensus site, TGTGGT.

Chromatin Immunoprecipitation Screen—The Chromatin immunoprecipitation screen was modified from the previously described strategy (19, 20). Chromatin was prepared from 10^8 control U937 cells transduced with LXIN or cells transduced with LXIN expressing the FLAG-tagged RUNX1 DNA-binding domain. The fixation was performed using 1% formaldehyde for 15 min at room temperature on a rocker platform. Fixation was terminated by adding glycine to 0.125 M and washing twice in phosphate-buffered saline. Lysis and sonication to 500-bp fragments were performed as described previously (19, 20). Chromatin samples were diluted 20-fold in immunoprecipitation buffer (0.1 M KCl, 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Tween 20) and incubated with anti-FLAG M2 agarose (Sigma) at 4 °C, on an end-over-end platform, overnight. After extensive washing with immunoprecipitation buffer, complexes were eluted from beads using 100 μ g/ml of FLAG peptide (Sigma), according to the manufacturer's instructions. Samples were de-cross-linked at 65 °C, treated with proteinase K, and end-repaired as described previously (19, 20). DNA fragments were purified with a QIAquick spin kit (Qiagen, Valencia, CA) and cloned into TOPO blunt vectors (Invitrogen) (19, 20). Equal volumes of TOPO ligation samples were transformed into DH5 α and plated on LB agar containing kanamycin (50 μ g/ml). Sequence from clones was obtained using a T7 primer.

Chromatin Immunoprecipitation—Aliquots of chromatin samples prepared as described above were immunoprecipitated using FLAG M2 agarose beads (Sigma) or anti-AML1 (Calbiochem) as appropriate. Immunoprecipitated samples were prepared for PCR as described and analyzed using primers for the PKC promoter (forward primer, ATCCATTGGTCATTCTGCA; reverse primer, TATTGATCTACTGAAATCCTCTCTC) or actin promoter (forward primer, GAGCACA-GAGCCTCGCCTTT; reverse primer, AGACAAAGACCCGCGGTT) (18). Amplification was performed using titanium *Taq* polymerase (Clontech) at thermocycler settings of: one cycle at 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, and 68 °C for 1 min. Aliquots were analyzed by agarose gel electrophoresis.

Electrophoretic Mobility Shift Analyses—Complementary oligomers, including the putative RUNX1-binding motif (CCAAGAATCTTCTCTGTGGT GGAGTTAGAGACATA), were annealed and labeled with digoxigenin dUTP (Roche). The probe was incubated with U937 cell extracts for 15 min at room temperature and loaded onto a 6% non-reducing mobility shift gel (Invitrogen). Probe was transferred to Nylon membrane, positively charged (Roche), cross-linked using a Stratalkiner (Stratagene, La Jolla, CA), and developed using a digoxigenin wash and buffer chemiluminescence (Roche). Competitor analyses were performed using 50 \times unlabeled double-stranded oligomers. Wild-type oligomers were identical to the probe. GCTAGC replaced the RUNX1 consensus TGTGGT in mutant oligomers.

Quantitative RT-PCR—Quantitative RT-PCR was performed using an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). *PKC β* primers (forward, GACCATGGACCGCTGTACTTTG; reverse, GAAGAACAGACCGATGGCAATTTCT), probe (CTTGAACCGGCCGACTTGCTGGA), actin primers (forward, AGAGATGGCCACGGCTGCTT; reverse, GGACTCCATGCCAGGAAGGAA), and probe (CACCATTGGCAATGACGGTTCCGC) were designed using Primer Express software (Applied Biosystems). Reactions were prepared using Taqman Universal PCR Master Mix (Applied Biosystems). Relative transcript levels represent $2^{-\Delta\Delta Ct}$ calculations.

Western Blot—Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol). Immunoblots were probed in Tris-buffered saline containing 0.15% Tween 20 and 3% milk, using anti-FLAG (Sigma), anti-RUNX1 (Ab-1; Calbiochem), anti-*PKC β* (sc-210; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-actin (Santa

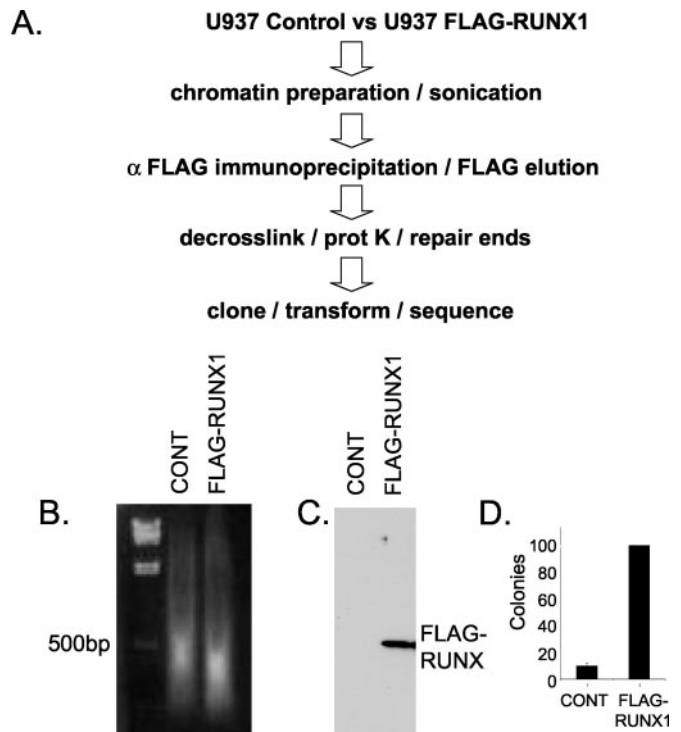


FIG. 1. Chromatin immunoprecipitation screen. *A*, schematic of FLAG ChIP screen. *B*, agarose gel demonstrating equivalent sonication of chromatin from U937 cell lines: LXIN control (*CONT*) and FLAG-RUNX1 LXIN. *C*, immunoprecipitation Western blot showing purification and elution of FLAG-RUNX1 protein from fixed and sonicated chromatin samples. Sonicated chromatin samples from U937 cell lines were incubated overnight with FLAG-agarose beads. Beads were washed in immunoprecipitation buffer and eluted with FLAG peptide. Immunoblot analysis was performed on eluted samples using an anti-RUNX1 antibody. *D*, DH5 α colony counts showing enrichment of fragments from FLAG-RUNX1 chromatin relative to control chromatin. DNA fragments prepared from eluted complexes were cloned and transformed into DH5 α . Counts from FLAG-RUNX1 samples were normalized to 100 colonies ($n = 4$).

Cruz Biotechnology) followed by secondary antibodies conjugated to horseradish peroxidase. Bands were visualized using Supersignal Fento (Pierce).

Luciferase Assays—U937 cells (10^6) from LXIN, *RUNX1*, or *RUNX1* DN lines were electroporated (200 V, 1000 microfarads) with firefly luciferase reporter vectors and SV40-*Renilla* luciferase expression vectors to control for transfection efficiency. Twenty-four hours after transfection, luciferase assays were performed using the dual-luciferase reporter assay system (Promega).

Apoptosis Assays—Pure cell line populations expressing the appropriate genes were obtained by selection on 1 mg/ml G418 (for LXIN or *PKC* LXIN) and cell sorting (for MigR1 or *RUNX1* DN MigR1) was performed using a FACS Vantage cell sorter (Becton Dickinson). U937 cells (10^6) were plated on six-well plates. Samples were treated with 10 nM PMA for 12 h and analyzed for annexin binding using Apop-TACS flow cytometry kit (R&D Systems, Minneapolis, MN) and streptavidin-allophycocyanin antibodies (Caltag Laboratories, Burlingame, CA). Samples were analyzed on a FACSCalibur (BD Biosciences). Histograms of annexin-positive cells represent the propidium iodide-negative population. TNF α was measured by enzyme-linked immunosorbent assay (R&D Systems).

RESULTS

Chromatin Immunoprecipitation Screen—To identify novel, direct RUNX1 gene targets, we modified a recently described, unbiased ChIP screen (Fig. 1A) (19, 20). We stably expressed a FLAG-tagged version of the RUNX1 DNA-binding domain in U937 myeloid cells using a bicistronic LXIN retroviral vector. The system of anti-FLAG immunoprecipitation and FLAG peptide elution allows for the gentle isolation of protein complexes in a single precipitation (26). Control LXIN cells and FLAG-

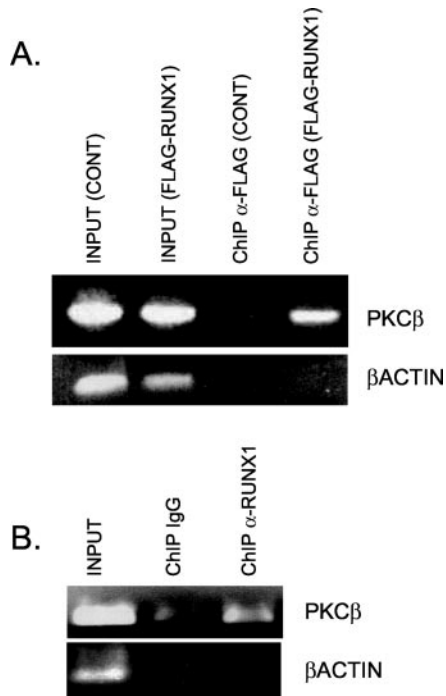


FIG. 2. Chromatin immunoprecipitation analysis showing interaction of RUNX1 with the *PKCβ* promoter. Samples were analyzed by PCR using primers amplifying *PKCβ* or actin promoter sequence. **A**, ChIP analysis of U937 cell lines used in the screen described in Fig. 1 (*CONT* versus *FLAG-RUNX1*). Immunoprecipitation was performed using anti-FLAG antibodies, and elution was performed using FLAG peptide. **B**, ChIP analysis of endogenous RUNX1 in wild-type U937 cells. Immunoprecipitation was performed using control IgG or anti-RUNX1 antibodies, and elution was performed using 1% SDS, 0.1 M HCO₃.

RUNX1 LXIN cells were fixed with formaldehyde, and chromatin was prepared. Samples were sonicated to equivalent sizes of ~500 bp (Fig. 1B). Chromatin immunoprecipitation was performed using anti-FLAG agarose beads, and RUNX1-chromatin complexes were eluted using FLAG peptide. This allowed for effective pull-down and elution of FLAG-RUNX1 from formaldehyde-fixed and sonicated samples (Fig. 1C). RUNX1-chromatin complexes were then processed to allow DNA fragments to be cloned. Transformation of DH5α with cloned fragments demonstrated a 10-fold enrichment in clones from cells expressing FLAG-RUNX1 compared with controls (Fig. 1D). This is consistent with enrichment for RUNX1 target-DNA fragments by the FLAG immunopurification.

***PKCβ* Is a Direct *RUNX1* Target**—We sequenced 10 clones derived from the FLAG-RUNX1 cell line and five clones from the control cell line. Three clones from the FLAG-RUNX1 line appeared to be from promoter regions, a frequency comparable with that of previously described cloning strategies (19). Furthermore, eight of the 10 clones from the FLAG-RUNX1 cell line represented unique genomic loci, whereas only one of five control clones was unique (data not shown). We pursued in more detail the most intriguing clone, which corresponded to bases -265 to -1003 of the promoter for the *PKCβ* gene (27).

PKCβ is expressed in myeloid cells and is associated with a well characterized apoptotic pathway, making it a potentially important leukemogenic target. To validate the FLAG-RUNX1 association with the *PKCβ* promoter, we performed conventional ChIP-PCR analyses. First, we repeated the ChIP using anti-FLAG immunoprecipitation and FLAG peptide elution, using primers designed from the cloned *PKCβ* promoter fragment. *PKCβ* promoter sequences were amplified specifically from the FLAG-RUNX1 samples, confirming the enrichment

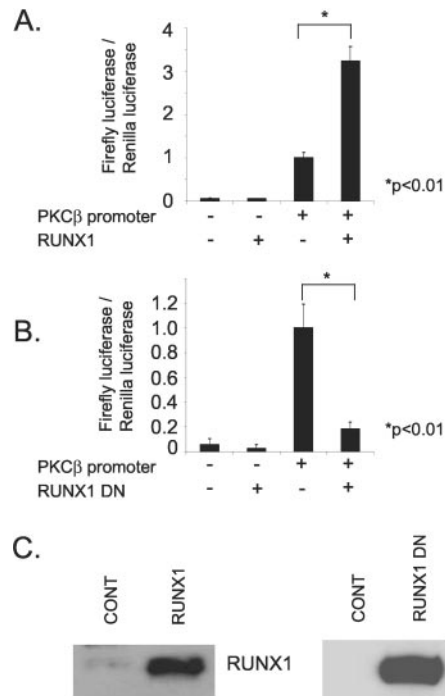


FIG. 3. Luciferase reporter assays indicating RUNX1 responsiveness of the *PKCβ* promoter. U937 cell lines indicated were transiently transfected with a firefly luciferase reporter control vector (-) or a reporter downstream of the *PKCβ* promoter (+). A vector expressing *Renilla* luciferase was co-transfected to control for transfection efficiency. Normalized firefly luciferase/*Renilla* luciferase values are shown. **A**, luciferase assays performed on U937 cell lines stably transduced with LXIN (-) or RUNX1 LXIN (+) ($n = 3$). **B**, luciferase assays performed on U937 cell lines stably transduced with LXIN (-) or dominant-negative RUNX1 (*RUNX1 DN*) (+) ($n = 3$). **C**, immunoblots from U937 cells demonstrating expression of the wild-type and dominant-negative RUNX1 proteins.

for these sequences by the FLAG immunopurification strategy (Fig. 2A). No enrichment of the control β -actin promoter was observed. To rule out differences in chromatin preparations as a cause of the positive ChIP results, we performed immunoprecipitations on a single chromatin sample from the cell line expressing FLAG-RUNX1. Anti-FLAG antibodies enriched for *PKCβ* promoter sequences significantly more than control rabbit IgG (data not shown). These PCR results indicate that FLAG-RUNX1 associates with the chromatinized *PKCβ* promoter in U937 cells.

We next performed immunoprecipitations on chromatin samples from wild-type U937 cells using anti-RUNX1 antibodies to identify an association between endogenous RUNX1 and the endogenous *PKCβ* gene. PCR analysis confirmed that immunoprecipitation of endogenous RUNX1 enriches chromatin fragments from the *PKCβ* promoter, in comparison to a control immunoprecipitation (Fig. 2B). This demonstrates that endogenous RUNX1 associates with the chromatinized *PKCβ* promoter of wild-type U937 cells.

Localization of the *RUNX1* Binding Site in the *PKCβ* Promoter—The observed association of RUNX1 with sequences upstream of *PKCβ* suggested that the promoter might be RUNX1-responsive. We, therefore, cloned the *PKCβ* promoter upstream of a firefly luciferase reporter gene and transfected control U937 cells and cells overexpressing RUNX1 or dominant-negative RUNX1 (Fig. 3C). RUNX1 increased luciferase expression by greater than 3-fold (Fig. 3A). Conversely, transfection of cells expressing dominant-negative RUNX1 resulted in reduced luciferase expression (Fig. 3B). These changes demonstrate that the *PKCβ* promoter confers RUNX1 responsiveness on a heterologous reporter gene and are consistent with

to PMA (31–35). U937 or HL60 cells treated with PMA undergo apoptosis (31–36). Cell lines deficient in PKC β are resistant to PMA-induced apoptosis, but the phenotype can be restored by ectopic expression of PKC β (31–35). Events downstream of PKC β may include production of TNF α , stimulation of ceramide synthesis, and activation of caspases (33, 36). Thus, a previously characterized PMA-PKC β -apoptosis pathway is available for functional analysis of RUNX1.

We expressed DN RUNX1 in U937 cells by retroviral transduction with the MigR1 vector and obtained pure populations by cell sorting. We hypothesized that these cells would have defective PKC β pathways and would be resistant to PMA-induced apoptosis. In agreement with previous studies, we found that control cells treated with PMA readily underwent apoptosis (Fig. 6A) (31–36). By contrast, we found that cells expressing DN RUNX1 were protected from apoptosis (Fig. 6, A and B). Similar results were obtained with stable U937 cell lines produced using LXIN and RUNX DN LXIN retroviral

vectors and selected on G418-containing media (data not shown). Resistance to apoptosis was correlated with a significant reduction in TNF α (from 4.04 ± 1.35 to 1.55 ± 0.35 ng/ml, $p < 0.02$), consistent with TNF α functioning downstream of PKC β (data not shown). Additionally, treatment of either control cells or cells expressing RUNX DN with TNF α induced apoptosis (data not shown). This demonstrates that cells expressing the RUNX DN protein retain an intact apoptotic pathway. Furthermore, this finding suggests that the blunted apoptotic phenotype results from altered PMA signaling. These findings are consistent with a defective PKC β pathway in cells expressing DN RUNX1.

Rescue of Apoptosis—The correlation between the expression of dominant-negative RUNX1 and a defect in the PKC β apoptotic pathway suggests that reduced PKC β expression could be the direct cause of this defect. To determine whether the reductions in PKC β produced the resistance to PMA, we stably expressed PKC β in U937 cells and transduced these cells with MigR1 control retrovirus or the dominant-negative RUNX1 retrovirus. We hypothesized that the apoptotic phenotype of cells expressing dominant-negative RUNX1 would be restored by PKC β expression. Indeed, following PMA treatment, we observed wild-type levels of apoptosis in the DN RUNX1 cells ectopically expressing PKC β (Fig. 6, A and B). Immunoblot analysis confirmed that PKC β protein levels reflect the sensitivity to PMA (Fig. 6C). This indicates that the reduction in apoptosis resulting from expression of dominant-negative RUNX1 is a direct result of reduced PKC β expression.

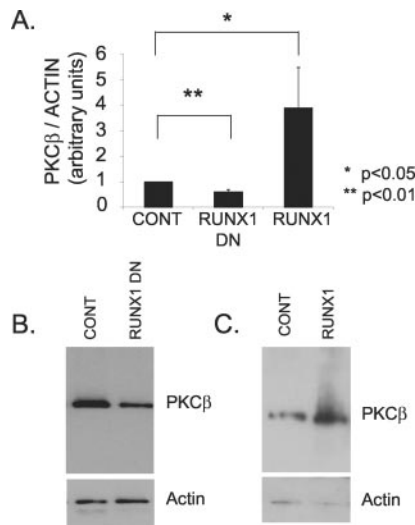
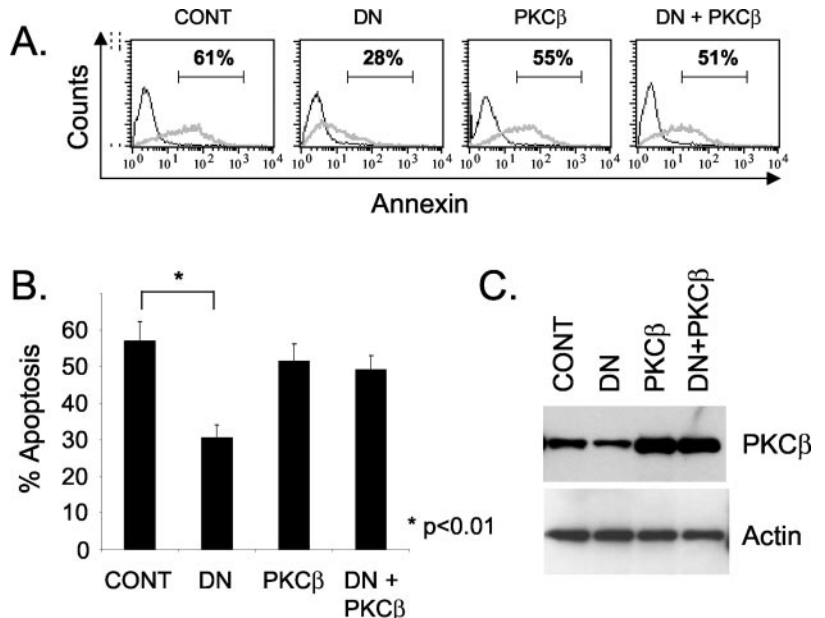


FIG. 5. Expression studies indicating effects of RUNX1 activity on endogenous PKC β levels. A, quantitative PCR analysis of PKC β expression in U937 cells expressing the RUNX1 DN ($n = 4$) or cells overexpressing wild-type RUNX1 ($n = 3$). -Fold changes of PKC β normalized to β -actin are shown. B, immunoblots of PKC β from U937 cells expressing the DN RUNX1 protein or, C, cells overexpressing wild-type RUNX1.

DISCUSSION

Our ChIP screen revealed that RUNX1 binds the PKC β promoter in myeloid cells. Candidate gene approaches and differential expression screens have not previously identified PKC β as a RUNX1 target gene in myeloid cells. ChIP is especially well suited to target identification because it is an unbiased biochemical technique. Targets are revealed without a prior need to identify affected pathways. Furthermore, the indirect effects of transcription factors are filtered because ChIP is based on DNA-binding properties instead of an ability to disrupt cellular biology. Additionally, prior knowledge of regulatory elements is not required, and elements are not assumed to fall within convenient proximal promoter regions. Regulatory regions are potentially revealed whether they are positioned at promoters, within distant enhancer elements, or

FIG. 6. Dominant-negative RUNX1 (RUNX1 DN) protects U937 cells from PMA-induced apoptosis. A, annexin staining. U937 cell lines expressing LXIN or LXIN-PKC β were transduced with MigR1 or RUNX1 DN MigR1 retrovirus. Twenty-four hours after transduction, cells were sorted and treated for 12 h with vehicle (*thin line*) or 10 nM PMA (*bold line*). Cells were stained with annexin-biotin/avidin-allophycocyanin. Histograms show annexin-positive, propidium-iodide-negative (*i.e.* non-necrotic) cells. Percentages represent apoptotic cells following PMA treatment. B, bar graphs of the percentage of apoptotic cells ($n = 4$); *, $p < 0.01$. C, cell extracts were analyzed by immunoblotting with anti-PKC and anti-actin antibodies.



even within introns. In support of the identification of *PKC β* as a novel RUNX target gene, we have shown that the *PKC β* gene promoter is activated by RUNX1 and contains a canonical binding site to which RUNX1 specifically binds. This binding site is sufficient for RUNX1 activation of a heterologous promoter. Mutation of the RUNX1-binding site in the context of the full-length *PKC β* promoter does not fully eliminate RUNX1 responsiveness of the promoter (data not shown), indicating that additional RUNX1-binding sites likely contribute to the regulation of this gene. Nevertheless, it is clear that endogenous RUNX1 binds to the endogenous *PKC β* gene and that *PKC β* gene activity is regulated by RUNX1. Interestingly, microarray analysis of lymphoid leukemia specimens recently revealed *PKC β* as a marker in samples from patients with the *TEL-AML1* (i.e. *TEL-RUNX1*) translocation but not other forms of acute lymphoid leukemia (37).

The *PKC β* pathway has been studied extensively in myeloid cells. Several myeloid lines, including a U937-derived line, express reduced levels of *PKC β* (38–40). This deficiency results in blunted PMA-induced apoptosis (31–35). *PKC β* is the only deficient member of the PKC family identified in these cells, and the apoptotic phenotype can be restored by ectopic expression of *PKC β* (31–35). Our demonstration that RUNX1 regulates *PKC β* provides a plausible and novel mechanism to explain previous observations linking RUNX1 to apoptosis. For example, the RUNX1-opposing fusion core-binding factor β -smooth-muscle myosin heavy chain inhibits apoptosis in myeloid cells (41). Furthermore, reduction in the RUNX1-interfering AML1-ETO oncoprotein in Kasumi cells results in apoptosis, suggesting that the oncoprotein protects cells from death-inducing background genes present in these lines (42). The present finding that cells expressing DN RUNX1 essentially recapitulate the phenotype of *PKC β* -deficient U937 cells and that this can be rescued by *PKC β* supports the hypothesis that *PKC β* regulation underlies at least some of the effects of RUNX1 on apoptosis.

The link between RUNX factors and apoptosis may not be limited to RUNX1 in myeloid cells, because loss of RUNX3 function produces gastric carcinoma, possibly by inhibiting the apoptotic response to transforming growth factor β (3). The link is complicated, however, because in some settings interference with RUNX activity has been associated with increased apoptosis (43), and for unknown reasons dominant-negative RUNX1 in the form of the RUNX DNA-binding domain sometimes behaves differently than AML1-ETO (29). In these settings, the role of *PKC β* regulation may be overshadowed by competing phenotypic effects of other RUNX target genes.

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