A Chromatin Immunoprecipitation Screen Reveals Protein Kinase Cβ 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 Cβ as a direct RUNX1 target gene and demonstrate that endogenous RUNX1 binds the chromatinized protein kinase Cβ promoter of U937 cells. A phylogenetically conserved RUNX1-binding site within the PKCβ promoter binds RUNX1 in electrophoretic mobility shift analyses and confers RUNX1 responsiveness on a heterologous promoter. Changes in RUNX1 activity affect endogenous protein kinase Cβ expression, and a dominant-negative form of RUNX1 protects U937 cells from apoptotic stimuli previously shown to be dependent on protein kinase Cβ. This protection can be reversed by the ectopic expression of protein kinase Cβ. Together these findings demonstrate that protein kinase Cβ 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 member, 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) 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 (PKCβ) as a direct RUNX1 target. Changes in RUNX1 activity affect PKCβ expression and a well characterized, PKCβ-dependent apoptotic pathway of myeloid cells. Thus, RUNX1 regulation of PKCβ 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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** The abbreviations used are: ChIP, chromatin immunoprecipitation; PKCβ, protein kinase Cβ; DN, dominant-negative; AML, acute myeloid leukemia; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift analysis.
Chromatin Immunoprecipitation Screen—The Chromatin immunoprecipitation screen was modified from the previously described strategy (19, 20). Chromatin was prepared from 10^6 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 μg/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 cloning vectors (Invitrogen). The PKCβ promoter construct encompassing bases −7 to −1003, with respect to the transcriptional 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 GCCCTTC in place of the RUNX1 consensus site, TGTGGT.

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, ATCCATGGTCACTTGCA, reverse primer, TATGGACTCTGTAATCTTCTCTC, actin promoter (forward primer, GAGGACAAGCCTGGCCGTT), (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. Reaction products were analyzed by agarose gel electrophoresis.

Electrophoretic Mobility Shift Analyses—Complementary oligomers, including the putative RUNX1-binding motif (GAGCTCCCTAATTCCTTCGCATGGGTAGGACAGTACCATCG), (19) were cloned into pGL2 basic vector (Promega). 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. DH5α 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 DH5α. 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 Femto (Pierce).

Luciferase Assays—U937 cells (10^6) from LXIN, RUNX1, or RUNX1 DN lines were electroporated (200 V, 1,000 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).

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-
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 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...
the observed association between RUNX1 and the PKCβ promoter in the ChIP assay.

The cloned PKCβ promoter fragment has one motif (positioned at −549 to −544 with respect to the transcriptional start site) that perfectly matches the RUNX1 consensus-binding site (TG(T/C)GGT) (27). Additionally, five sites deviating by 1 bp are scattered throughout the cloned region. To identify sequences potentially bound by RUNX1, we aligned the human and mouse sequences (Fig. 4A). Of the six potential regulatory regions, only the TGTGGT consensus is conserved between species, suggesting that it is potentially a transcription factor-binding site. To test this hypothesis, a 35-bp probe including the conserved TGTGGT motif was incubated with U937 cell nuclear extract and subjected to electrophoretic mobility shift analysis (EMSA). The extract resulted in a shift of the probe, demonstrating an interaction of a factor in U937 cells with this region (Fig. 4B). The shifted probe could be competed by an excess of unlabeled oligonucleotide but not by an oligonucleotide carrying a mutation of the TGTGGT motif to GCTAGC. Incubation of the probe with extracts plus anti-RUNX1 antibody supershifted the probe, confirming that RUNX1 is, indeed, able to interact with the TGTGGT motif (Fig. 4C). Furthermore, a tandem repeat of the wild-type oligomer placed upstream of a heterologous promoter-reporter construct is sufficient to confer RUNX1 responsiveness in transfected cells (Fig. 4D). The mutant oligomer that failed to bind RUNX1 in the EMSA lacks this activity in the transfection assay. These findings confirm the ChIP results by demonstrating that RUNX1 interacts with and activates a binding site from the PKCβ promoter.

PKCβ Expression Studies—The validation of RUNX1 interactions with the PKCβ promoter suggested that the expression of endogenous PKCβ should be affected by changes in RUNX1 activity. We used the MigR1 retrovirus to overexpress wild-type RUNX1 or a DN protein (RUNX1 DN) consisting of the DNA-binding domain without the co-regulator interaction domain (25, 28–30). The retroviruses express the RUNX genes and green fluorescent protein from a bicistronic message allowing for the production of unselected, multiclonal populations of transduced cells by sorting. Quantitative RT-PCR analysis indicated that DN RUNX1 reduced PKCβ expression by greater than 3-fold (Fig. 5A). PKCβ A expression and are consistent with RUNX1 regulating PKCβ expression and are consistent with RUNX1 regulating the PKCβ promoter.

Inhibition of Apoptosis—The regulation of PKCβ levels by RUNX1 suggests that RUNX1 function should influence the PKCβ pathway. Numerous studies in myeloid cells have shown that PKCβ functions in the apoptotic response
to PMA (31–35). U937 or HL60 cells treated with PMA undergo apoptosis (31–36). Cell lines deficient in PKCβ are resistant to PMA-induced apoptosis, but the phenotype can be restored by ectopic expression of PKCβ (31–35). Events downstream of PKCβ may include induction of TNFα, stimulation of ceramide synthesis, and activation of caspases (33, 36). Thus, a previously characterized PMA-PKCβ-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 PKCβ 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 TNFα (from 4.04 ± 1.35 to 1.55 ± 0.35 ng/ml, p < 0.02), consistent with TNFα functioning downstream of PKCβ (data not shown). Additionally, treatment of either control cells or cells expressing RUNX DN with TNFα 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 PKCβ pathway in cells expressing DN RUNX1.

**Rescue of Apoptosis**—The correlation between the expression of dominant-negative RUNX1 and a defect in the PKCβ apoptotic pathway suggests that reduced PKCβ expression could be the direct cause of this defect. To determine whether the reductions in PKCβ produced the resistance to PMA, we stably expressed PKCβ 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 PKCβ expression. Indeed, following PMA treatment, we observed wild-type levels of apoptosis in the DN RUNX1 cells ectopically expressing PKCβ (Fig. 6, A and B). Immunoblot analysis confirmed that PKCβ 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 PKCβ expression.

**DISCUSSION**

Our ChIP screen revealed that RUNX1 binds the PKCβ promoter in myeloid cells. Candidate gene approaches and differential expression screens have not previously identified PKCβ 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
even within introns. In support of the identification of PKCβ as a novel RUNX target gene, we have shown that the PKCβ 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 PKCβ 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 PKCβ gene and that PKCβ gene activity is regulated by RUNX1. Interestingly, microarray analysis of lymphoid leukemia specimens recently revealed PKCβ 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 PKCβ pathway has been studied extensively in myeloid cells. Several myeloid lines, including a U937-derived line, express reduced levels of PKCβ (38–40). This deficiency results in blunted PMA-induced apoptosis (31–35). PKCβ is the only deficient member of the PKC family identified in these cells, and the apoptotic phenotype can be restored by ectopic expression of PKCβ (31–35). Our demonstration that RUNX1 regulates PKCβ 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 PKCβ-deficient U937 cells and that this can be rescued by PKCβ supports the hypothesis that PKCβ 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 growth of this organ (35). Li et al. have shown that RUNX1 regulates expression of the PKCβ gene and that the PKCβ gene activity is regulated by RUNX1. Interestingly, microarray analysis of lymphoid leukemia specimens recently revealed PKCβ 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).

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