Platelet Protein Kinase C-θ Deficiency With Human RUNX1 Mutation

PRKCQ Is a Transcriptional Target of RUNX1

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Objective—Mutations in the hematopoietic transcription factor RUNX1 cause thrombocytopenia and impaired platelet function. In a patient with a heterozygous mutation in RUNX1, we have described decreased platelet pleckstrin phosphorylation and protein kinase C-θ (PKC-θ, gene PRKCQ) associated with thrombocytopenia, impaired platelet aggregation, and dense granule secretion. Little is known regarding regulation of PKC-θ in megakaryocytes/platelets. We have addressed the hypothesis that PRKCQ is a direct transcriptional target of RUNX1.

Methods and Results—In a chromatin immunoprecipitation assay using megakaryocytic cells, there was RUNX1 binding in vivo to PRKCQ promoter region −1225 to −1056 bp containing a RUNX1 consensus site ACCGCA at −1088 to −1069 bp; an electrophoretic mobility shift assay showed RUNX1 binding to the specific site. In RUNX1 overexpression studies, PKC-θ protein expression and promoter activity were enhanced; mutation of RUNX1 site showed decreased activity even with RUNX1 overexpression. Lastly, PRKCQ promoter activity and PKC-θ protein were decreased by short interfering RNA knockdown of RUNX1.

Conclusion—Our results provide the first evidence that PRKCQ is regulated at the transcriptional level by RUNX1 in megakaryocytic cells and a mechanism for PKC-θ deficiency associated with RUNX1 haploinsufficiency. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: Platelets ■ PKC-θ ■ PRKCQ ■ RUNX1 ■ megakaryocytes ■ transcriptional regulation
Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed using HEL cells (1×10⁶) treated with PMA for 24 hours and the ChIP-IT kit (Active Motif, Carlsbad, CA) as described. Sheared fragments were immunoprecipitated with anti-RUNX1 antibody (sc-8564x, Santa Cruz Biotechnology, Santa Cruz, CA) or the negative control IgG antibody supplied in the kit. We amplified 3 regions (−665 to −478 bp, −1225 to −1056 bp, and −1468 to −1277 bp) of the PRKCQ promoter with specific primers.

EMSA

Nuclear extracts were prepared from PMA-treated HEL cells according to Dignam et al. Nuclear protein-DNA interactions were performed using PKC-θ, PKC-δ, and PKC-ε as DNA-binding proteins and the double-stranded oligo containing RUNX1 sites (underlined) as DNA probe. EMSA was performed using PKC-θ wild-type (WT) probes used to examine RUNX1 binding to each consensus site in (bold) and their mutants generated by deletions in RUNX1 sites (underlined) as follows: WT probe I (−585 to −566) with site I, 5'-CTGGGGTACCAGTTCA-3', and its mutant 5'-CTGGGGTATTGAGGTTCA-3'; WT probe II (−618 to −599) with site II, 5'-GAAAGTGATACCCCGAAC-3', and its mutant 5'-GAAAGTGATTGGCACCGAC-3'; WT probe III (−1088 to −1070) with site III, 5'-ATGGGACCCCGCCA-CCTGGCC-3', and its mutant 5'-ATGGGACCCCGCAGCTTTGGC-3'; and WT probe IV (−1442 to −1423) with site IV, 5'-ACCTTCAGGGGTTCTTTTAG-3', and its mutant 5'-ACCTTCAGGGGTTCTTTTAG-3'. Three micrograms of nuclear extract and 50 fmol of labeled probe were used in binding reactions performed on ice for 30 minutes. Oligonucleotide competitors were added 30 minutes before the labeled probe was added. For supershift assays, RUNX1 antibody (sc-8563x) or control IgG (Santa Cruz Biotechnology) was preincubated with nuclear protein before labeled probe was added. In addition, EMSA was performed with recombinant RUNX1 protein (200 ng) to see its binding to PKC-θ probes. Binding reaction was performed on ice for 1 hour in a buffer containing 0.6 mmol/L HEPES, pH 8.0, 1 mmol/L dithiothreitol, 0.01% Triton X-100, 2% glycerol, 5 µg/µL bovine serum albumin, and 100 mmol/L NaCl. For supershift studies, anti-RUNX1 antibody (sc-8564x) or control IgG (Santa Cruz Biotechnology) was preincubated for 30 minutes on ice with recombinant RUNX1 protein before the addition of labeled probe. Binding complexes were separated by electrophoresis on native 5% Tris-borate-EDTA gels (Bio-Rad) and detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Construction of Luciferase Reporter Plasmids

The PRKCQ promoter region (−1085 to −206 bp) was amplified by a standard PCR from human genomic DNA using modified primers incorporating with restriction sites Xhol in the forward primer and HindIII in the reverse primer. The PCR product was cloned into pCR 2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA). Recombinant HEL cells, RUNX1 protein, RUNX1 expression plasmid RUNX1−pCmv6−XL4, empty expression vector pCmv6−XL4, and transfection agent Turbofectin 8.0 were from Origene Technologies (Rockville, MD).

Cell Lines and Cell Culture

Human erythroleukemia (HEL) cell line from American Type Cell Culture (Rockville, MD) was cultured in RPMI-1640 medium (Mediatech) in the presence of 10% fetal bovine serum and antibiotics (Mediatech). During induction, HEL cells were grown in 10 mmol/L PMA.

We have reported detailed studies on a patient with inherited thrombocytopenia; decreased platelet aggregation, secretion, and GPIIb-IIIa activation; impaired phosphorylation of plectin and myosin light chain; and decreased platelet PKC-θ (protein and mRNA) associated with a mutation in RUNX1. We have recently shown that RUNX1 regulates platelet PKC-θ signaling, and pleckstrin and myosin light chain phosphorylation; platelet function, very little is known about its transcriptional regulation, and its association with a mutation in RUNX1. We have previously described the clinical presentation and underscored the complex nature of altered RUNX1 binding to DNA but without direct DNA contact. RUNX1 mutations are associated with familial, autosomal-dominant thrombocytopenia; platelet dysfunction; and predisposition to acute leukemia. On the basis of the decreased platelet PKC-θ in our patient, we hypothesized that platelet/megakaryocyte PKC-θ is regulated at the transcriptional level by RUNX1 and constitutes the mechanism for PKC-θ deficiency. Despite the important role of PKC-θ signaling in platelet function, very little is known about its transcriptional regulation in MK cells. In the present studies, we provide the first evidence that PKC-θ is a direct transcriptional target of RUNX1. We have recently shown that RUNX1 regulates ALOX12, MYL9, and PF4. The present studies extended this to PRKCQ and underscored the complex nature of alterations in platelets/megakaryocytes in human RUNX1 haploinsufficiency.

Materials and Methods

Patient Information

We have previously described the clinical presentation and studies in this 24-year-old white male, documenting decreased agonist-stimulated platelet aggregation, secretion, GPIIb-IIIa activation, and plecstrin and myosin light chain phosphorylation; platelet PKC-θ level was decreased. The patient has a single point mutation in RUNX1, in intron 3 at the splice acceptor site for exon 4, leading to a frameshift with premature termination in the conserved Run homology domain.

Materials

All chemicals, including phorbol 12-myristate 13-acetate (PMA), were purchased from Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). GoTaQ Green PCR Master Mix, luciferase reporter vectors pGL3-Basic and pRL-TK, and the Dual Luciferase Assay System were from Promega (Madison, WI). GoTaq Green PCR Master Mix, luciferase reporter vectors pGL3-Basic and pRL-TK, and the Dual Luciferase Assay System were from Promega (Madison, WI). Nuclear protein-DNA interactions were performed using PKC-θ wild-type (WT) probes used to examine RUNX1 binding to each consensus site in (bold) and their mutants generated by deletions in RUNX1 sites (underlined) as follows: WT probe I (−585 to −566) with site I, 5'-CTGGGGTACCAGTTCA-3', and its mutant 5'-CTGGGGTATTGAGGTTCA-3'; WT probe II (−618 to −599) with site II, 5'-GAAAGTGATACCCCGAAC-3', and its mutant 5'-GAAAGTGATTGGCACCGAC-3'; WT probe III (−1088 to −1070) with site III, 5'-ATGGGACCCCGCCA-CCTGGCC-3', and its mutant 5'-ATGGGACCCCGCAGCTTTGGC-3'; and WT probe IV (−1442 to −1423) with site IV, 5'-ACCTTCAGGGGTTCTTTTAG-3', and its mutant 5'-ACCTTCAGGGGTTCTTTTAG-3'. Three micrograms of nuclear extract and 50 fmol of labeled probe were used in binding reactions performed on ice for 30 minutes. Oligonucleotide competitors were added 30 minutes before the labeled probe was added. For supershift assays, RUNX1 antibody (sc-8563x) or control IgG (Santa Cruz Biotechnology) was preincubated with nuclear protein before labeled probe was added. In addition, EMSA was performed with recombinant RUNX1 protein (200 ng) to see its binding to PKC-θ probes. Binding reaction was performed on ice for 1 hour in a buffer containing 0.6 mmol/L HEPES, pH 8.0, 1 mmol/L dithiothreitol, 0.01% Triton X-100, 2% glycerol, 5 µg/µL bovine serum albumin, and 100 mmol/L NaCl. For supershift studies, anti-RUNX1 antibody (sc-8564x) or control IgG (Santa Cruz Biotechnology) was preincubated for 30 minutes on ice with recombinant RUNX1 protein before the addition of labeled probe. Binding complexes were separated by electrophoresis on native 5% Tris-borate-EDTA gels (Bio-Rad) and detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

The PRKCQ promoter region (−1085 to −206 bp) was amplified by a standard PCR from human genomic DNA using modified primers incorporating with restriction sites Xhol in the forward primer and HindIII in the reverse primer. The PCR product was cloned into pCR 2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA), digested with Xhol and HindIII enzymes, and cloned into appropriate sites of luciferase vector, pGL3-Basic. The primer sequences of Wt construct were as follows: forward at 618 to 599 with site II, 5'-GAAAGTGATACCCCGAAC-3', and its mutant 5'-GAAAGTGATTGGCACCGAC-3'; WT probe III (−1088 to −1070) with site III, 5'-ATGGGACCCCGCCA-CCTGGCC-3', and its mutant 5'-ATGGGACCCCGCAGCTTTGGC-3'; and WT probe IV (−1442 to −1423) with site IV, 5'-ACCTTCAGGGGTTCTTTTAG-3', and its mutant 5'-ACCTTCAGGGGTTCTTTTAG-3'. Three micrograms of nuclear extract and 50 fmol of labeled probe were used in binding reactions performed on ice for 30 minutes. Oligonucleotide competitors were added 30 minutes before the labeled probe was added. For supershift assays, RUNX1 antibody (sc-8563x) or control IgG (Santa Cruz Biotechnology) was preincubated with nuclear protein before labeled probe was added. In addition, EMSA was performed with recombinant RUNX1 protein (200 ng) to see its binding to PKC-θ probes. Binding reaction was performed on ice for 1 hour in a buffer containing 0.6 mmol/L HEPES, pH 8.0, 1 mmol/L dithiothreitol, 0.01% Triton X-100, 2% glycerol, 5 µg/µL bovine serum albumin, and 100 mmol/L NaCl. For supershift studies, anti-RUNX1 antibody (sc-8564x) or control IgG (Santa Cruz Biotechnology) was preincubated for 30 minutes on ice with recombinant RUNX1 protein before the addition of labeled probe. Binding complexes were separated by electrophoresis on native 5% Tris-borate-EDTA gels (Bio-Rad) and detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences).
RUNX1 expression plasmid, RUNX1-pCMV6-XL4, or empty vector pCMV6-XL4 (1 µg each), along with an internal control, pRL-TK containing the Renilla luciferase gene (20 ng), using the Turbofectin 8.0 transfection reagent. 

**Results**

Whole cell lysates (30 to 40 µg) from cotransfected HEL cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to Odyssey nitrocellulose membranes (Li-Cor Biosciences), and probed with the antibodies against RUNX1 (sc-8563), PKC-θ (sc-1875), and actin (sc-47778) from Santa Cruz Biotechnology. Specific protein expressions were detected with infrared-labeled secondary antibodies using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Bioinformatics

Potential binding sites for transcription factors were analyzed by the computer program TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html).

**Binding of RUNX1 to the PRKCO Promoter by ChIP**

Analysis of the PRKCO promoter region (2075 bp from the ATG) using TFSEARCH revealed 5 RUNX1 consensus sites: −577 to −572 (site I), −610 to −605 (site II), −1081 to −1076 (site III), −1313 to −1308 (site IV), and −1437 to −1432 (site V) (Figure 1A). We performed immunoprecipitations on chromatin samples from PMA-treated HEL cells using an anti-RUNX1 antibody to identify an endogenous interaction between RUNX1 and the PRKCO gene. PCR primers were designed to amplify PKC-θ regions −665 to −478 bp (containing sites I and II), −1225 to −1056 bp (containing site III), and −1468 to −1277 bp (containing sites IV and V). These primers were used to amplify HEL cell chromatin enriched by anti-RUNX1 antibody. Only region −1225 to −1056 bp (containing site III) was enriched by RUNX1 antibody (Figure 1B). These observations indicate that RUNX1 binds in vivo to site III but not to other sites under the conditions studied.

**Binding of RUNX1 to Consensus Sites on the PRKCO Promoter by EMSA**

EMSA was performed using HEL cell nuclear extracts and WT probe I, which contains RUNX1 consensus site I (Figure 2A). Protein binding occurred with probe I (Figure 2A, lane 2). Binding was not altered by competition with the mutant probe with mutation of RUNX1 site (lane 3) but was lost by competition with wild-type probe (lane 4).
competition with unlabeled Wt probe (lane 4). Binding to the Wt probe was inhibited by competition with RUNX1 antibody (lanes 5 and 6 show decreasing antibody concentration) but not by IgG (lane 7).

Figure 2B shows nuclear protein binding to probe containing consensus site II (lane 2). This binding was competed by excess unlabeled probe (lane 3) but not by RUNX1 antibody (6 µg) (lane 4). Competition studies were performed with 2, 4, and 6 µg of antibody, but binding was not affected. It was not altered by normal IgG (lane 5). Lane 6 shows no effect on binding by competition with excess unlabeled mutant probe with the RUNX1 site mutated. These findings suggest that the observed protein binding to the probe with site II is not due to RUNX1, and the findings are in line with those of ChIP (Figure 1).

Figure 2C shows protein binding to probe III with RUNX1 consensus site III (lane 1); this was abolished by competition with excess unlabeled probe (lane 2). Binding was inhibited by RUNX1 antibody (lanes 3 and 4) but not by IgG (lane 5). DNA-protein binding was not altered by competition with the mutant probe (lane 6). Lane 7 shows labeled probe alone. These data indicate that RUNX1 binds to site III. EMSA performed on probes with site IV or V showed no protein binding (data not shown). Together, these results indicate that RUNX1 binds to sites I and III, as measured by EMSA.

### Binding Studies With Recombinant RUNX1

To further establish that RUNX1 binds to PKC-θ probes I and III, we performed studies using recombinant RUNX1 protein. Figure 3A shows RUNX1 binding to probe I, containing site I (lane 2). This binding was competed by excess unlabeled probe (lane 3), unaffected by normal IgG (lane 4), but supershifted by anti-RUNX1 antibody (lane 5). Similar results were obtained with probe containing site III (Figure 3B). These data provide further support that RUNX1 binds to sites I and III.

### Discussion

The major novel finding in our studies is that the transcription factor RUNX1 regulates the PRKCQ gene in MK cells. In...
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Figure 5. Inhibition of PKC-θ protein and promoter by RUNX1 siRNA in HEL cells. A, Immunoblotting of HEL cell lysates showing β-actin, RUNX1, and PKC-θ. RUNX1 siRNA inhibited PKC-θ and RUNX1 protein and promoter activity. B, EMSA with nuclear extracts and recombinant RUNX1 (Figure 1), suggesting in vivo binding of RUNX1 to site III. EMSA with nuclear extracts and recombinant RUNX1 (Figures 2 and 3) showed RUNX1 binding to site III. Mutation of RUNX1 site III abolished promoter activity, indicating its functional importance. Overexpression of RUNX1 increased RUNX1 and PKC-θ proteins and promoter activity, which was lost on mutation of site III (Figure 4). RUNX1 siRNA inhibited PKC-θ protein expression and PRKCQ promoter activity (Figure 5). Together, these findings indicate that PKC-θ is regulated at the transcriptional level by RUNX1 and that site III is crucial for transcriptional control of PRKCQ in MK cells. The studies presented here were performed in HEL cells treated with PMA to induce MK differentiation, a widely used model for studies on megakaryocyte biology.

That these findings are directly relevant to platelet PKC-θ expression is supported by the findings in our patient with RUNX1 haploinsufficiency, which showed that PKC-θ protein and mRNA are decreased in platelets, the primary cells. In addition, we have shown that pleckstrin phosphorylation was decreased in the platelets, which provides strong evidence for a functional consequence of the diminished PKC-θ expression. The present studies provide a cogent explanation for decreased platelet PKC-θ associated with RUNX1 haploinsufficiency. Previous studies in U937 cells have shown PKC-β to be a direct RUNX1 target; platelet PKC-β was normal in our patient.

RUNX1 haploinsufficiency is associated with familial thrombocytopenia, predisposition to acute leukemia, impaired megakaryopoiesis, and impaired platelet function on activation. Mice lacking RUNX1 have a complete absence of fetal liver-derived hematopoiesis and impaired MK maturation; Runx1 haploinsufficiency is associated with decreased platelet number. Multiple lines of evidence link PKC to critical aspects of MK differentiation. Phorbol esters activate PKC and induce progenitor cells to differentiate along megakaryocyte lines and express megakaryocyte/platelet proteins.

In human progenitors, PKC-θ exhibits a lineage-restricted expression being expressed in megakaryocytes and erythroblasts but not granulocytes/monocytes. Jacquel et al have proposed that specific PKC isoforms, including PKC-θ, may not be able to induce MK differentiation alone but that more than 1 PKC isoform may be required for the differentiation process. The specific role of PKC-θ in megakaryopoiesis and in platelet formation needs to be defined. Interestingly, dominant-negative inhibition of PKC-θ delays cell cycle progression in vascular endothelial cells.

Platelet functional abnormalities constitute a hallmark of RUNX1 mutations. We postulate that the deficiency of platelet PKC-θ arising secondary to the RUNX1 haploinsufficiency contributes to the functional defect shown in our patient’s platelets, including in aggregation, secretion, αIIbβ3 activation, and cytoskeletal reorganization, all of which have been noted in studies in murine PKC-θ-deficient platelets. Moreover, pleckstrin is a major substrate phosphorylated by PKC in platelets and this phosphorylation was impaired in our patient. Pleckstrin-deficient mouse platelets showed marked defects in PKC-mediated exocytosis of dense and α granules, αIIbβ3 activation, actin assembly, and aggregation, which provide further support for a potential role of PKC-θ in the platelet dysfunction observed in our patient. Although we propose that PKC-θ deficiency contributes to the functional defect in RUNX1 haploinsufficiency, the magnitude of PKC-θ’s role remains to be delineated, particularly because RUNX1 regulates several other genes also recognized to regulate platelet responses.

As shown by us, these include ALOX12 (12-lipoxygenase), MYL9 (myosin light chain), PF4 (platelet factor 4), and possibly others. Overall, the concept emerging is that RUNX1 haploinsufficiency-associated platelet dysfunction and thrombocytopenia arise from the interactions involving multiple genes. Studies in RUNX1 haploinsufficiency provide an opportunity to unravel the role in platelets/MK of various RUNX1-regulated genes and proteins, including those that are currently not recognized to have a role in platelets but are downregulated.

In summary, our studies reveal that PRKCQ is regulated by RUNX1 in megakaryocytes/platelets and provide an explanation for the decreased PKC-θ expression in RUNX1 haploinsufficiency. RUNX1 dysregulation of PRKCQ in megakaryocytes is an important aspect of the abnormal platelet production and function associated with human RUNX1 mutations and is an area for further investigation to unravel the mechanisms leading to defects in platelet production and function.

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Disclosures
None.

References


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