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Nuclear Factor-κB Regulates Expression of Platelet Phospholipase C-β2 (*PLCB2*):

Mao: NF-κB Regulates Platelet PLC-β2

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Summary

Phospholipase C (PLC)-β2 (gene *PLCB2*) is a critical regulator of platelet responses upon activation. Mechanisms regulating of PLC-β2 expression in platelets/MKs are unknown. Our studies in a patient with platelet PLC-β2 deficiency revealed the *PLCB2* coding sequence to be normal and decreased platelet PLC-β2 mRNA, suggesting a defect in transcriptional regulation. *PLCB2* 5'- upstream region of the patient revealed a heterozygous 13 bp deletion (−1645/−1633 bp) encompassing a consensus sequence for nuclear factor-κB (NF-κB). This was subsequently detected in 3 of 50 healthy subjects. To understand the mechanisms regulating PLC-β2 we studied the effect of this variation in the *PLCB2.* Gel-shift studies using nuclear extracts from human erythroleukemia (HEL) cells or recombinant p65 showed NF-κB binding to oligonucleotide with NF-κB site; in luciferase reporter studies its deletion reduced *PLCB2* promoter activity. *PLCB2* expression was decreased by siRNA knockdown of NF-κB p65 subunit and increased by p65 overexpression. By immunoblotting platelet PLC-β2 in 17 healthy subjects correlated with p65 (r=0.76, p=0.0005). These studies provide the first evidence that NF-κB regulates MK/platelet PLC-β2 expression. This interaction is important because of the major role of PLC-β2 in platelet activation and of NF-κB in processes, including inflammation and atherosclerosis, where both are intimately involved.

Keywords

Megakaryocytess; nuclear factor-κB; phospholipase C-β2; platelet function defect; platelets

Introduction

Phospholipase C-β2 (PLC-β2, gene *PLCB2*) catalyzes the hydrolysis of phosphatidylinositol bisphosphate to yield two intracellular mediators, inositol 1,4,5 trisphosphate (Ins 1,4,5 P3)

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and diacylglycerol, and plays a major role in platelet responses to activation of Gαq-coupled receptors by agonists, including ADP, thrombin and thromboxane A2 (1, 2). PLC-β2 is the most abundant of the platelet PLC-β isozymes (3). Little is known about the regulation of this enzyme in platelets/MKs. We have previously described a patient with platelet deficiency of PLC-β2 (OMIM Accession Number 604114) associated with impaired aggregation, secretion, calcium mobilization and pleckstrin phosphorylation on platelet activation (4, 5). The production of Ins 1,4,5 P3 and phosphatidic acid were decreased on thrombin activation (5). The index case showed a decrease in platelet PLC-β2 both by immunological methods and activity (3). Agonist-induced Ca^{2+} mobilization, PLC-β2 mRNA and PLC-β2 protein were normal in neutrophils suggesting a lineage specific defect in PLC-β2 expression (6). PLC-β2 coding region was normal in the patient, however, platelet PLC-β2 mRNA was decreased by ~50% (6), suggesting a defect in the transcriptional regulation of *PLCB2*.

To understand the mechanisms regulating platelet PLC-β2 expression, we characterized ~2000 bp of 5'-upstream region of *PLCB2* in the patient. These studies showed in the PLC-β2 5'-upstream region a 13-bp deletion (−1645/−1633) that encompasses a consensus-binding site for nuclear factor-κB (NF-κB), a family of transcription factors that promote or repress expression of numerous genes (7–11). This deletion was subsequently found in some healthy control subjects. NF-κB plays a major role in a wide range of cellular events including response to cytokines, inflammation, atherogenesis and megakaryocytic differentiation (7–11). Platelets and megakaryoctes (MK) express NF-κB proteins and one of them BcL-3 is induced on platelet activation (12). There are 5 members of this family (Rel A (p65), Rel B (p68), c-Rel, p50 and p52), which form various complexes with different DNA-binding specificities (10, 11). NF-κB complexes are sequestered in the cytoplasm, related to their binding to inhibitory proteins called IκBs. NF-κB activation involves phosphorylation by kinase Iκκ of IκB, degradation of the inhibitory subunit, and subsequent translocation of NF-κB to the nucleus where it binds to the promoter regions of specific genes. Numerous genes have been identified that are NF-κB targets and these participate in cell proliferation, inflammatory and immune responses, antiapoptotic responses and other cell functions (9–11, 13). It is unknown whether PLC-β2 is regulated by NF-kB in platelets and MKs

In the present study, we provide the first evidence that platelet PLC-β2 expression is regulated by NF-κB via a NF-κB consensus site located in a 13 bp deletion detected in *PLCB2* 5' upstream region. In addition, we studied the effect of two other variations detected in the *PLCB2* 5' upstream region of the patient. These studies advance understanding of the regulation of PLC-β2, a major player in platelets/ MKs.

Methods

Materials.

Taq polymerase, deoxyribonucleotide triphosphate (dNTP), PGL3-Basic vector, the Renilla luciferase control vector, and Dual-Luciferase Reporter Assay System kit were purchased from Promega Biotech (Madison, WI). All oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Antibodies against NF-κB (p65 subunit), TFII-I,

PLC-β2, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin 3'-end DNA labeling kit and LightShift™ Chemiluminescent EMSA kit was purchased from Pierce (Rockford, IL). Phorbol 12-myristate 13-acetate (PMA) and carbacyclin were obtained from Biomol (Plymouth Meeting, PA).

Patient information.

Details of the platelet studies in the patient and her son have been described (3, 5, 6). Both subjects have markedly impaired aggregation, dense granule secretion and Ca^{2+} mobilization upon platelet activation. Ins 1,4,5 P3 and pleckstrin phosphorylation were diminished in both. The healthy control subjects were recruited from donors at the Thrombosis Research Center and denied taking any medications known to affect platelet function.

DNA Isolation and Promoter Sequence Analysis by PCR.

Blood (42.5 ml) was collected from donors into 7.5 ml acid citrate dextrose (ACD) solution (71.4 mM citric acid, 85 mM sodium citrate-dihydrate, 11.1 mM dextrose) and supplemented with carbacyclin (30 nM). Leukocyte genomic DNA was extracted by proteinase K-phenol-chloroform method (14). PCR amplification of DNA was performed in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). Primers were designed based on GenBank PLC-β2 genomic sequence (Supplemental Table 1). The strategies used to define the alterations in the *PLCB2* 5' upstream of the patient are described in Supplemental Methods.

Promoter-Reporter Constructs and Mutagenesis.

Genomic fragments of 1648, 1631 and 1791 nucleotides of PLC-β2 5'-upstream sequence were obtained by PCR amplification of genomic DNA using primers designed against the *PLCB2* promoter sequence in GenBank. Details are provided in the Supplemental Materials. Mutant constructs with the 13 nt deletion (−1633 to −1645 nt), 7 nucleotide deletion (−1184 to −1190 nt) and the combined 13 and 7 nucleotide deletions using the Quick Change Site-Directed Mutagenesis Kit (Stratagene Inc., La Jolla, CA).

Luciferase Reporter Assays.

Human erythroleukemia (HEL) cells (American Type Culture Collection, Rockville, MD) was cultured in RPMI medium supplemented with 10% fetal bovine serum (Gemini Bioproducts Inc., Calabasas, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, at 37°C, with 5% carbon dioxide (CO_2). HEL cells (3×10^6) were transfected (15 μg construct) by electroporation and stimulated with PMA (30 nM) to induce megakaryocytic transformation. Control vector PRL-thymidine kinase (TK)-Luc (Promega Corp.), expressing the Renilla luciferase, was cotransfected to correct for transfection efficiency. Activity was measured in cell extracts using Dual Luciferase Assay System (Promega).

Electrophoretic mobility shift assay (EMSA).

EMSA was performed using nuclear extracts from HEL cells treated with PMA (30 nM) for 48 h and with recombinant NF-κB p65 (Active Motif, Carlsbad, CA). Oligonucleotides with

the consensus NF-κB DNA sequence (−1652 5'-CTGTGCTGGGAATTCCCTTAGCTCC-3' −1628) (15) and its deletion form (5'-CTGTGCTTTAGCTCC −3') and the site mutated form (5'-CTGTGCTCCCTTAAGGGTTAGCTCC-3'), and oligonucleotides with the TFII-1 consensus sequence (−1205 5'-GCACACGGAGGAGAGAGGAGAGCGCAGTGG-3' −1176) along with a 7 nt deletion (−1190 to −1184) form (5'-GCACACGGAGGAGAGCGCAGTGG-3') were end-labeled using the Biotin 3'-end DNA labeling Kit (Pierce). (The NF-κB and TFII-I consensus sequence are underlined). DNAs cross-linked to the membrane were detected using horse-radish peroxidase-conjugated streptavidin (LightShift™ chemiluminescent EMSA kit). Additional details are provided under Supplemental Materials. For the super-shift assay, the reaction mixture was combined with 1–2 μg of anti-p65 (Santa Cruz, SC-7151X) or 1 μg TFII-I antibody (Santa Cruz, sc-9943X) and incubated for 30 min before adding probes.

Studies with NF-κB Small Interfering (si)-RNA.

Small interfering RNA, a pool of a 3 target-specific 20–25 nt siRNA designed to knock down NF-κB p65 gene expression, and nonspecific RNA were from Santa Cruz Biotechnology. PMA (30 nM) was added to HEL cells 2 h prior to transfection to induce megakaryocytic transformation. PLC-β2 −1648/Luc plasmid DNA and siRNA were cotransfected with Lipofectamine™ 2000 (Invitrogen). Promoter activity was measured at 72 h. For analysis of mRNA expression, HEL cell RNA was reverse transcribed and cDNA was PCR amplified (30 cycles) with gene specific primers and each cycle was performed at 94° C for 30 sec, 58°C for 30 sec, and 72°C for 60 sec. PCR products were analyzed on 1% agarose gels. The primers used for PCR amplification were: PLC-β2 sense, 5'-AGTATTGCCGGACGATCT TTGG-3'; PLC-β2 antisense, 5'-TCAAAGGAGAC GAACTTGGTGG-3'; β-actin sense, 5'- GTCACCAACTGGGACGACATGGAG-3'; β-actin antisense, 5'-CTTGATCTTCATTGTG CTGGGTGC-3'. NF-κB primer pair was obtained from Santa Cruz (SC-44212-PR). For immunoblotting, lysates were prepared using M-per™ Mammalian Protein Extraction Reagent (Pierce Chemical) with proteinase inhibitors. The primary antibodies used were rabbit polyclonal antibodies against NF-κB p65, PLC-β2, and β-actin (Santa Cruz, CA). They were detected using peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI). The blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Bedford, MA), and detected using a Image Analyzer (Fuji Systems, Stamford, CT).

For studies on Ca^{2+} mobilization, HEL cells transfected with NF-kB p65 siRNA or control siRNA (Santa Cruz) were cultured in medium containing PMA (30 nM). Growth medium was replaced with RPMI containing 10% FBS and 20 mM HEPES, pH 7.3 after 72 h. Cells were loaded with 5 μM Fura-2/AM for 1 h at 37°C, washed and intracellular Ca^{2+} concentration was recorded following stimulation with thrombin (1 U/ml) with excitation at 340 nm and 380 nm, and emission monitored at 510 nm using an AB2 luminescence spectrophotometer (Spectronics Instruments, Rochester, NY) (16).

Effect of p65 overexpression on PLC-β2 expression.

The expression plasmid NF-kB p65-pCMV4 was obtained from Addgene (plasmid 21966; pCMV4 p65) (17). The 2.5 kb p65 cDNA was released with Hind III (Promega) and the

recircled empty pCMV4 vector DNA was used as control. HEL cells were transiently cotransfected using Lipofectamine 2000 (Invitrogen) with 1.6 μg of p65 expression plasmid p65-pCMV4 or empty pCMV4 vector and PLCβ2 promoter luciferase reporter constructs (wildtype or with 13 bp deletion). The cells were harvested 24 h for luciferase activity and immunoblotting.

Platelet PLC-β2 levels by immunoblotting in healthy subjects.

Platelet-rich plasma was prepared from blood collected into acid-citrate-dextrose (with 30 nM carbacyclin). Platelets were resuspended in Hepes buffer (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM Glucose, 10 mM Hepes (pH 7.4), 0.2% BSA) and washed twice. The platelet lysates were subjected to immunoblotting for PLC-β2, p65 and actin. The amounts were quantified by using standard curves based on serial protein amounts and normalized against actin.

Bioinformatics and statistical analyses.

Potential transcription factor binding sites on *PLCB2* promoter region were analyzed by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and TESS (http://www.cbil.upenn.edu/tess). Where shown, the data was analyzed by Student test.

All studies were performed after approval from the Temple University School of Medicine Institutional Review Board and after obtaining informed consent.

Results

Sequence variations in the PLCB2 5' upstream region of patient.

We sequenced 2165 bp of *PLCB2* 5' upstream region from ATG; compared to the GenBank sequence 3 variations were detected in the patient. The first was a heterozygous 13 bp deletion (−1645 to −1633 nt from ATG) (Fig. 1), noted in the patient and her son. Genomic DNA from the patient was PCR amplified using primers encompassing the region with the13 bp deletion (−1645/−1633). Initial studies in five healthy subjects showed a single band of expected size (Fig. 1A). The patient and her son showed 2 bands, consistent with a heterozygous defect (Fig. 1A). The first 10 nt of the 13 nt deletion constitute a palindrome (Fig. 1B). This 13 bp region was noted to have a consensus sequence (GGGAATTCCC) (−1645/−1636) for the transcription factor NF-κB (15) by TESS search. Subsequent sequencing studies in 50 healthy subjects (Supplemental Table 2) revealed this heterozygous 13 bp deletion in 3 subjects.

The second variation observed in the patient's *PLCB2* 5' upstream region was a homozygous 7 bp deletion (−1190 to −1184, AGGAGAG) in the patient; this was heterozygous in the son (Fig. 2). Computer based search revealed that this 7 bp region involves an overlapping consensus site for transcription factor TFII-I (−1194/−1189; AGAGAG) (18–20) (Fig. 2). PCR amplification of the region (−1150 to −1208) in the patient showed a single band of lower than expected size (Fig. 2A, lane P). Among the initial 5 control subjects, 3 (N1, N2, N3) showed only a single higher molecular weight band, one control subject showed only the smaller size band (N4) and one control subject showed both

(N5). These studies suggest that this 7 bp deletion is homozygous in the patient. Her son showed both bands consistent with a heterozygous state (not shown). Of note, in 50 control subjects, 29 and 19 subjects showed homozygous and heterozygous 7-bp deletion, respectively; only 2 showed the intact GenBank sequence (Supplemental Table 2). In the 3 control subjects with the 13 bp deletion, two of them had homozygous deletion of the 7-bp region (as noted in the propositus) and one was heterozygous. The expected number of individuals among the controls with a heterozygous 13-bp deletion and homozygous 7-bp deletion was estimated from Hardy-Weinberg equilibrium at 3.45%. It was 2.06% for the combination of the 13-bp deletion and heterozygous 7-bp deletion.

The third variation observed in the patient was a single base variation C to G at −325 bp. We have performed studies to characterize each of these variations in the *PLCB2* 5' upstream region.

DNA-Protein Binding Studies on PLCB2 Region −1652/−1628 bp with NF-κB Consensus Site.

We determined whether NF-κB binds to the *PLCB2* promoter in the region of the 13-bp deletion. EMSA was performed using PMA-treated HEL-cell nuclear extracts and oligonucleotide probes, both wildtype and with NF-κB site mutated (Fig. 3A). There was protein binding to wildtype probe (Fig. 3B, lane 1), which was competed by excess unlabelled wildtype probe (lane 2), but not by the mutant probe with NF-κB site deleted (lane 3) or mutated from GGGAATTCCC to CCCTTAAGGG (lane 4). No protein binding was observed with the two mutated probes with NF-κB site mutated (lanes 5 and 6). These studies indicate specific protein binding to the NF-κB site. Fig. 3C shows the effect of antibody against the p65 subunit (Rel A) of NF-κB on DNA-protein interaction. Lanes 1 and 2 show the extract and probe alone, respectively. Protein binding was observed (lane 3), which has competed by excess unlabelled probe (lane 4). Anti-p65 antibody inhibited the protein binding whether it was incubated with the nuclear extract before addition of the probe (lane 5) or along with the probe (lane 6). These studies indicate that NF-κB binds in the region evaluated. As additional evidence, we performed EMSA using recombinant p65 subunit of NF-κB (Fig. 3D). Protein binding was observed with wildtype probe (Lane 2) that was competed by excess unlabeled probe (lane 3). Anti-p65 antibody (Lane 4) but not nonspecific IgG (Lane 5) induced a supershift of the band. Competition with unlabelled probes with the NF-κB site deleted (Lane 6) or mutated (Lane 7) did not affect protein binding to wildtype probe. Together, the studies with HEL cell nuclear extracts and the recombinant p65 provide evidence of NF-κB binding to *PLCB2* region −1645/−1636.

EMSA Studies on PLCB2 Region −1205/−1176 with TFII-I Consensus Site.

These studies showed that TFII-I binds to this region. Figure 1 of Supplemental Materials shows EMSA using probes (−1205/−1176) with wildtype sequence and with the 7 bp (−1190/−1184) deletion, and HEL-cell nuclear extracts. DNA-protein complex was observed with the wildtype probe (lane 2), which was inhibited by excess unlabelled probe (lane 3) and by antibody against TFII-I (Lanes 4 and 5), but not by nonspecific IgG (Lane 6).

Effect of 13-bp (−1645/−1633) and 7 bp (−1190/−1184) Deletions on PLCB2 Expression.

To demonstrate a functional effect, we performed luciferase reporter assay in HEL cells treated with PMA to induce megakaryocytic transformation (21). In initial studies with 1648 bp of PLC-β2 5'-promoter region, truncation at −1631, which deletes the 13 bp region containing the NF-κB binding site, resulted in a ~25% decrease in activity (Fig. 4A). In further detailed studies, 1791 bp of the PLC-β2 upstream region, with and without deletions of the 13 (NF-κB) and the 7 bp (TFII-I) sequences, alone and in combination, was inserted upstream of the luciferase reporter and transfected into HEL cells. Promoter activity increased over time (16, 24, 48 h) with the wildtype (Fig. 4). Deletion of the 7 bp region with TFII-I sequence (−1184/−1190 bp) showed small non-significant decrease ($p>0.05$) (Fig. 4B). Deletion of the 13 bp region (−1633/−1645 bp) encompassing the NF-κB site was associated with a striking 36% ($p<0.05$) decrease in activity at 48 h, 19% at 24 h ($p<0.05$) and 22% at 16 h ($p<0.05$). In general, the largest decrease in activity (35–40%) at each time point was noted when both the 13 bp and 7 bp regions were deleted; however, at 48 h the decrease with 13 bp deletion alone was not different from that with the combination. These studies indicate that the 13 bp region with the NF-κB consensus sequence regulates PLC-β2 expression with a possible small effect of the 7 bp deletion region.

To determine whether PLC-β2 gene expression was upregulated during megakaryocytic differentiation, we performed studies in untreated and PMA-treated HEL cells using the wildtype construct. At 48 h promoter activity was ~5 fold higher and PLC-β2 protein was increased over 5 fold in PMA-treated cells (Not shown).

Studies on C/G Variation at −325.

The third variation observed in the patient was a C/G variation at position −325; the GenBank showed C at −325. This variation was seen in 2 of 3 control subjects studied. There was no difference in reporter activity with constructs with G or C at −325 (Fig. 4C).

Effect of siRNA Knockdown of NF-κB (p65) on PLCB2 Expression.

p65 siRNA, but not the control siRNA, decreased the mRNA levels of both PLC-β2 and p65 in HEL cells (Fig. 5A), with a corresponding decrease in both proteins (Fig. 5B and C) and in *PLCB2* promoter activity (Fig. 5D). To determine if the decreased *PLCB2* expression translated into a functional effect, we studied thrombin-induced rise in cytoplasmic Ca^{2+} level in cells loaded with Fura-2. It was decreased in p65 siRNA-treated cells but not with control siRNA (Fig. 5E); in parallel both PLC-β2 and p65 were decreased by immunoblotting (not shown).

Effect of p65 Overexpression on PLC-β2 Expression.

Overexpression of p65 markedly increased *PLCB2* promoter activity. No increase was noted following deletion of the region with NF-κB binding site (Fig. 6A), indicating that the specific site plays a role in the upregulation. Western blots showed upregulation of NF-κB p65 (Fig. 6B).

Platelet PLC-β2 and p65 in Healthy Subjects.

We measured platelet PLC-β2 and p65 by immunoblotting in 17 healthy subjects (12 males, 5 females); including 3 with the 13 bp (−1645 to-1633) deletion (1 heterozygous for 7 bp deletion and 2 homozygous) (Table 1). Quantification by immunoblotting with normalization against actin revealed a strong correlation between platelet PLC-β2 and p65 levels (r=0.76, p=0.0005) (Fig. 7B) consistent with the premise that NF-κB regulates PLC-β2. In the 3 subjects with the 13 bp deletion platelet PLC-β2 was not decreased (Fig. 7A) suggesting that this deletion was unlikely the major mechanism for the low platelet PLC-β2 in the patient.

Discussion

Our studies provide the first evidence that expression of platelet/MK PLC-β2, a major signaling protein involved in platelet activation, is regulated by transcription factor NF-κB. Studies in our patient with platelet PLC-β2 deficiency and her son revealed in the *PLCB2* 5'upstream region a heterozygous 13-bp deletion (−1645/−1633 bp), which encompasses a NF-κB binding site (Fig. 1). This deletion was subsequently detected in 3 of 50 control subjects. Our studies showed that p65 NF-κB subunit binds to the region with the 13bp deletion (Fig. 3) and regulates *PLCB2* promoter activity (Fig. 5). Moreover, siRNA knockdown of NF-κB p65 decreased PLC-β2 mRNA and protein in HEL cells (Fig. 5); in parallel, thrombin-stimulated Ca^{2+} response was blunted (Fig. 5E). p65 overexpression upregulated promoter activity, which was lost on deletion of the 13 bp region (Fig. 6). These studies provide evidence that NF-kB regulates PLC-β2 expression. This is strongly corroborated at the platelet level by the striking correlation between PLC-β2 protein and p65 protein in healthy subjects (Fig. 7B).

These findings are important because of the critical role of PLC-β2 in platelet responses to all Gαq-mediated agonists (1), and the role of NF-κB in diverse processes and states, including megakaryocytic differentiation, inflammation, atherosclerosis, diabetes and sepsis (7–11) (22, 23), where platelets are important players. For example, NF-κB activates numerous target genes including cytokines, chemokines, leukocyte adhesion molecules, and genes regulating cell proliferation and cell survival that contribute to atherogenesis (9–11, 13). Upregulation of PLC-β2 and related signaling mechanisms may be an important consequence and contribute to atherogenesis via enhanced platelet responsiveness. Studies in the ApoE-deficient mouse model of atherosclerosis indicate that hematopoietic PLC-β2 contributes to this process (24). NF-κB regulation of PLC-β2 may modulate the enhanced platelet responsiveness in inflammatory states via the NF-kB binding site. Of note, plasma levels of lipopolysaccharide (LPS), a ligand for pro-inflammatory toll-like receptor (TLR)-4 that activates NF-kB, are increased in DM (25); and platelets have TLR-4 (12). In recent studies using infusion clamps in healthy subjects we have shown that hyperglycemia - hyperinsulinemia increases circulating TLR-4, a receptor whose activation leads to upregulation of NFkB (26).

The role of NF-κB in MKs and platelets in inflammation and immunity is emerging (12). MKs and platelets contain nearly all NF-κB family members (27). Proliferating mouse MKs have an active NF-κB and it is modulated by Iκκ (28). Thrombopoietin-induced signaling in

these cells involves NF-κB and Iκκ (28), and thrombopoietin activates NF-κB and multiple signaling pathways in HepG2 cells (29). NF-κB has been implicated in megakaryocytic transformation of K562 and HEL cells by PMA (7, 8). PMA-treatment of human MKs upregulates p65 (Rel A), p50 and p52 (27). In line with this, our studies show that PMA upregulates PLC-β2 and p65 in HEL cells. Important to note, an effect on MKs translates to an effect in platelet levels - because the majority of platelet proteins come from the MK. Thus, we postulate that the observed strong relationship between platelet PLC-β2 and p65 (Fig. 7B) reflects an effect of NF-kB at the MK level. Moreover, expression profiling of platelets showed (30) upregulation of several genes and proteins by 24 hr of hyperglycemia-hyperinsulinemia, including glycogen synthase kinase 3 (GSK3); we believe these reflect effects at the MK level, manifested in the platelet.

Platelets are endowed with the NF-κB/I-κBα complex and Iκκ, and platelet activation leads to phosphorylation of I-κBα followed by its degradation (27, 31, 32). Inhibitors of NF-κB inhibit platelet aggregation, secretion, lamella formation and clot retraction (32). Overall, NF-κB appears to modulates platelet function (33) and is a major regulator of gene expression in megakaryocyes, including, as shown here, of PLC-β2. Platelets can process mRNA with activation-dependent protein synthesis, including of Bcl-3, an I-κB-α family member, and interleukin (IL)-1β (34, 35). Whether PLC-β2 levels are similarly enhanced by platelet activation is worthy of study.

With respect to our patient, *PLCB2* coding sequence was normal but platelet *PLCB2* mRNA was decreased (6), suggesting a defect in transcriptional regulation. Because the detected upstream 13-bp deletion was detected in some healthy subjects without a decrease in platelet PLC-β2, the patient's deficiency is likely related to other as yet undefined abnormalities in cis or trans elements that regulate basal *PLCB2* expression, although this may involve interaction with NF-kB, because transcription factors function in a combinatorial manner to regulate gene expression. NF-kB is upregulated in a host of acute and inflammatory states; we postulate that the identified NF-kB binding site regulates platelet *PLCB2* expression in the acute states, while other regulatory mechanisms critical to the basal levels are abnormal in our patient. Multiple mechanisms may regulate the cell expression of a protein under different conditions. Studies from our group and others have provided evidence that mutations in transcription factors may be the basis for inherited defects in platelet function; as for example heterozygous mutations in RUNX1 are associated with impaired expression of genes including *ALOX12, PF4, MYL9 and PRKQ* (36).

In summary, our studies provide evidence that NF-κB regulates expression of PLC-β2, a critical enzyme in platelet activation. Further studies are needed to assess the impact of this regulation on platelet responsiveness in various disease states associated with NF-kB activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

AKR designed the research study, performed the research and wrote the paper. GFM performed the research and contributed to the drafting of the paper. JJ performed some of the experiments and contributed to the drafting of the paper. SPK performed some of the experiments and contributed to the review of the paper. The authors gratefully acknowledge the excellent assistance of Denise Tierney in manuscript preparation.

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What is known on this topic?

- Phospholipase C (PLC)-β2 (gene *PLCB2*) is a critical regulator of platelet responses upon activation.
- Mechanisms regulating of PLC-β2 expression in platelets/ megakaryocytes are unknown.
- Platelets play a major role in hemostasis, thrombosis, inflammation and atherosclerosis.

What this paper adds:

- These studies provide the first evidence that transcription factor NF-κB regulates megakaryocyte/platelet PLC-β2 expression.
- This interaction is important because of the major role of PLC-β2 in platelet activation and of NF-κB in processes, including inflammation and atherosclerosis.

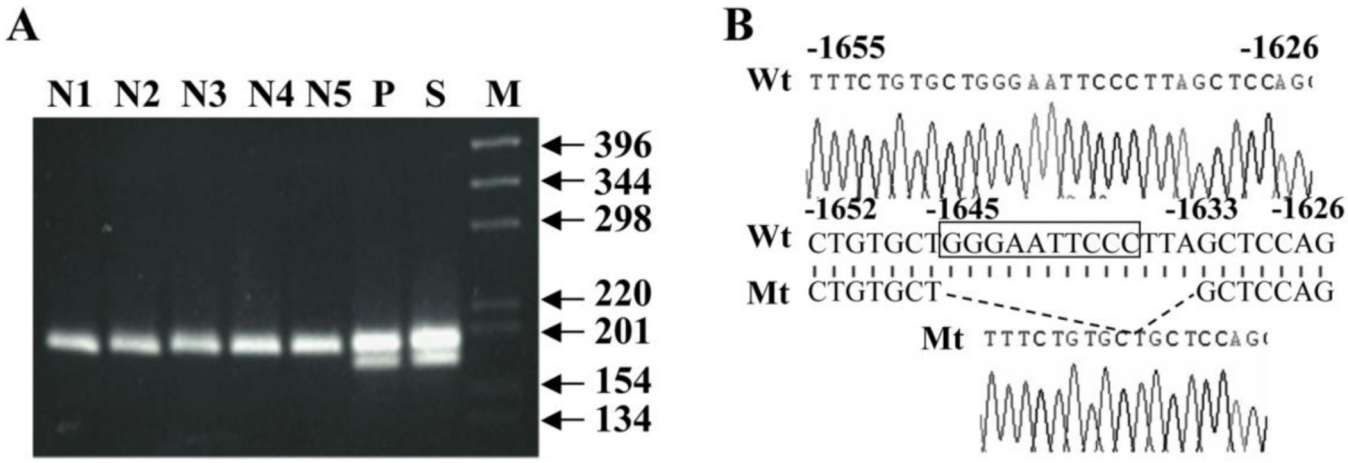

Figure 1. PCR amplification of *PLCB2* 5' upstream region showing 13 bp (−1645/−1633) deletion.

A. Genomic DNA from five normal subjects and the two patients was PCR amplified using primers corresponding to −1625/−1606 nt and −1791/−1774 nt (from ATG) in *PLCB2* 5' upstream region. Two PCR products of 186 bp (expected size) and 173 bp were noted in the patient (P) and son (S) with only a single 186 bp band in the five normal subjects (N1 to N5). Molecular markers are shown on the right. In subsequent studies a heterozygous 13 bp deletion was detected in 3 of 50 healthy subjects. **B.** Nucleotide sequence showing the wildtype (WT) allele and mutant allele with 13 bp deletion (−1633 to −1645). Box indicates NF-κB consensus binding site.

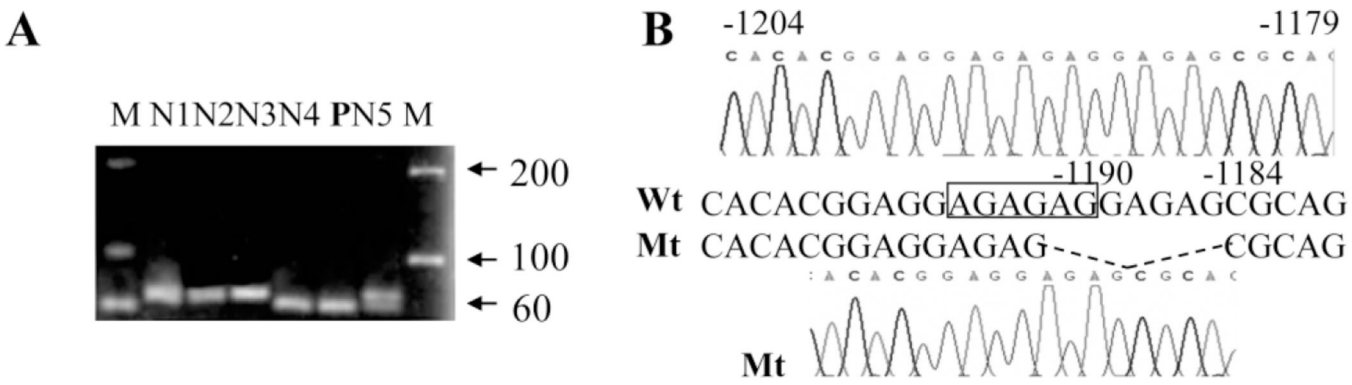

Figure 2. PCR amplification of *PLCB2* 5' upstream region showing the 7 bp (−1190/−1184) deletion.

A. Genomic DNA from five normal subjects and the patient was PCR amplified using primers corresponding to −1174/−1156 nt and −1226/−1208 nt of *PLCB2* 5' upstream. A WT single band of 71bp (expected size) was noted in the three normal subjects (N1 to N3). Two PCR products of 71 bp and 64 bp (7 bp deleted size) were noted in one healthy subject (N5). The patient (P) and one healthy subject (N4) showed a single 64 bp band. Molecular-mass markers (M) are shown on the right and left respectively. **B.** Nucleotide sequence showing the WT and variant allele with a 7 nt deletion (−1190 to-1184 nt from ATG). Box indicates TFII-I binding site.

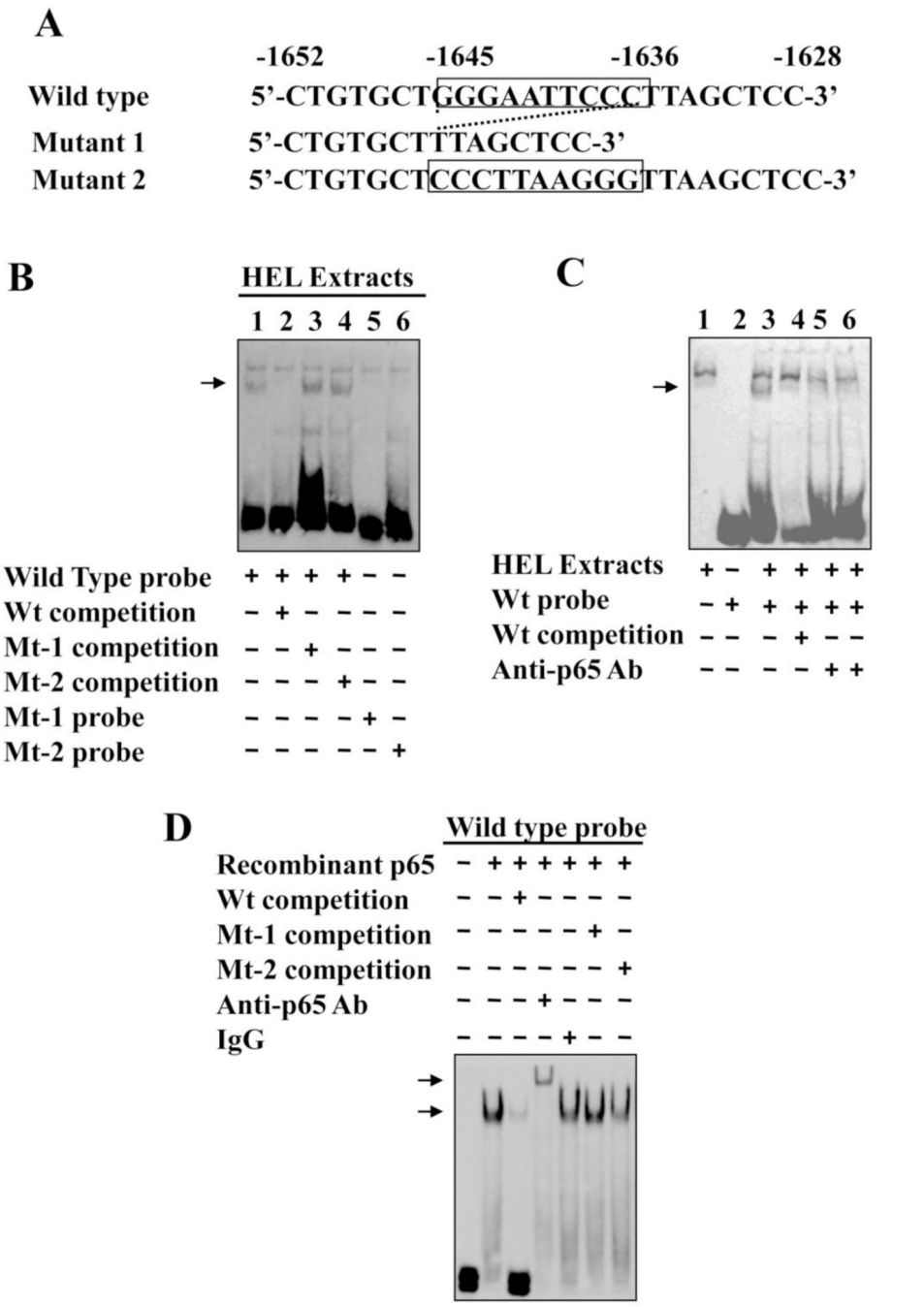

Figure 3. EMSA showing NF-κB binding to *PLCB2* region −1652/−1628.

A. DNA probes, wildtype and mutated, used in gel shift assays. Box shows the NF-κB consensus site. In Mutant 1 the 10 nt sequence was deleted. In Mutant 2 the consensus sequence was mutated. **B.** EMSA using HEL cell extracts and biotin-labeled probes. Lane 1, DNA-protein complex with WT probe. Lane 2, inhibition of complex by excess (200X) unlabelled WT probe but not by mutant 1 or 2 probes (lanes 3 and 4). Lane 5 and 6, absence of protein binding to mutant probes. **C.** Effect of NF-κB p65 antibody on protein binding. Lanes 1 and 2, extract and probe alone, respectively. Lane 3 shows DNA-protein binding

(arrow), which is competed by excess (200X) unlabelled probe in lane 4. Lanes 5 and 6, inhibition of complex by anti-p65 antibody, incubated with the nuclear extract before (lane 5) or along with the probe (lane 6). **D**. EMSA with recombinant human p65 protein. Lane 1, probe alone. Lane 2, formation of DNA-protein complex (lower arrowhead), which is competed by excess (200X) unlabelled probe in lane 3. Lane 4, super-shifted complex with p65 antibody (top arrowhead). Lane 5, lack of effect of non-specific IgG. Lanes 6 and 7, the complex was not inhibited by mutant probes.

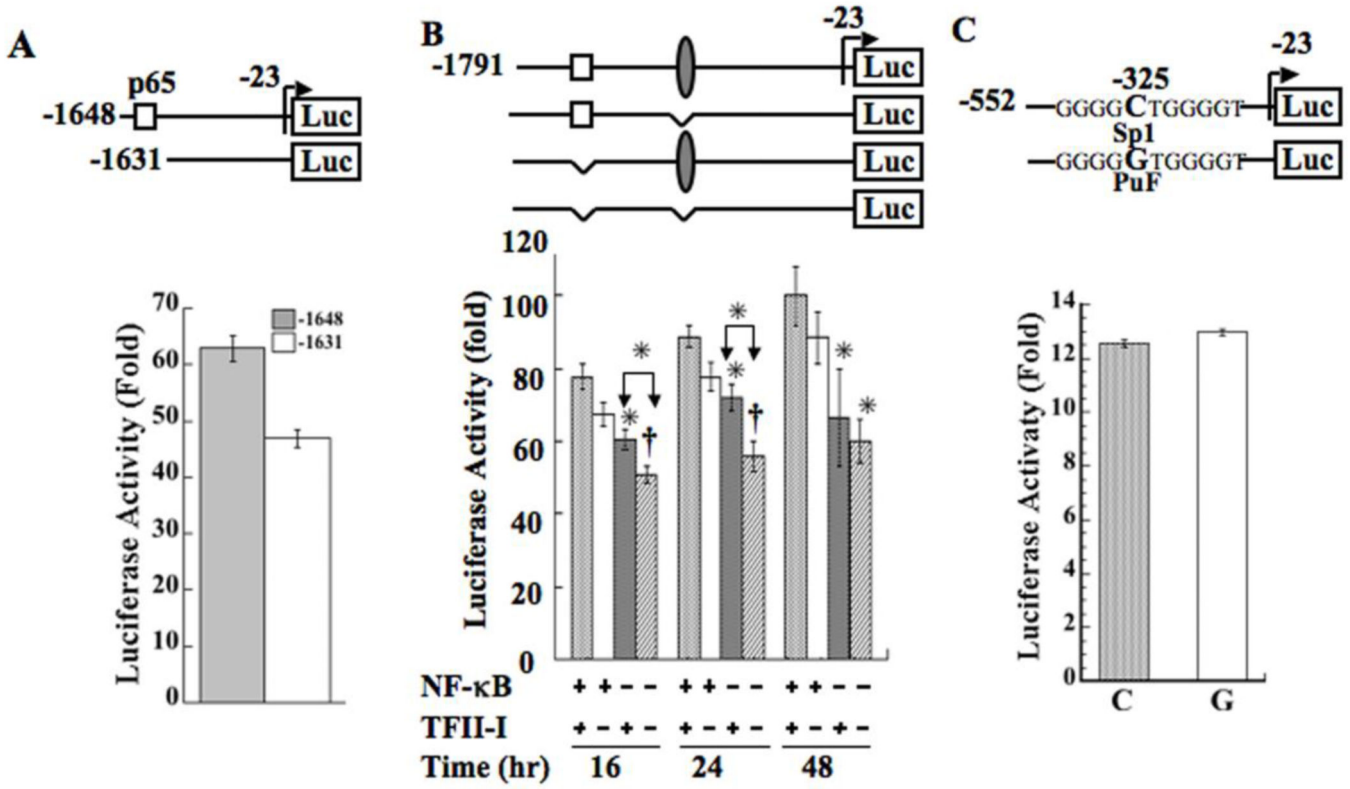

Figure 4. Luciferase reporter studies on *PLCB2* promoter.

PMA-treated HEL cells were transfected with *PLCB2* promoter reporter constructs shown along with pRL-TK control plasmid. **A.** Reporter studies with construct containing −1648/−23 *PLCB2* promoter region. Top panel, a schema of constructs used. Cells were transfected with luciferase reporter construct −1648/−23 region with NF-κB site (−1633 to −1645 nt) (open box) or with truncated construct −1631/−23 with this region eliminated. Shown luciferase activity at (means ± S.D) at 48 h from 3 experiments. **B.** Luciferase reporter studies with WT *PLCB2* promoter region −1791/−23, and with constructs with deletion of the 7-bp region with TFII-I site, with deletion of the 13-bp region with NF-κB site and with both sites deleted. Constructs used are shown at the top; the open box indicates the 13 nucleotides (−1645 to −1633 nt) with NF-κB site; the vertical ellipse represents the 7 nucleotides (−1190 to −1184 nt) with TFII-I site. The 13 or 7 nt deletions are shown by the break in line. The lower part shows reporter activity at 16, 24 and 48 h. (+) indicates presence of the site; (–) indicates deletion of the site. Shown are results from 4 experiments. The asterisks at the top of each bar show comparison of activity with WT construct: *p<0.05, †p<0.001. The asterisks above the arrowheads show comparison of 13 nt deletion alone with combined 13 nt and 7 nt deletions (p<0.05). **C.** Impact of C/G variation at −325 nt on *PLCB2* promoter activity. Constructs contained promoter region −552/−23 nt. The C/G variation encompassed predicted consensus sites for Sp1 and PuF. Top, schematic representation of constructs. Lower part, luciferase activity with C or G at −325 nt.

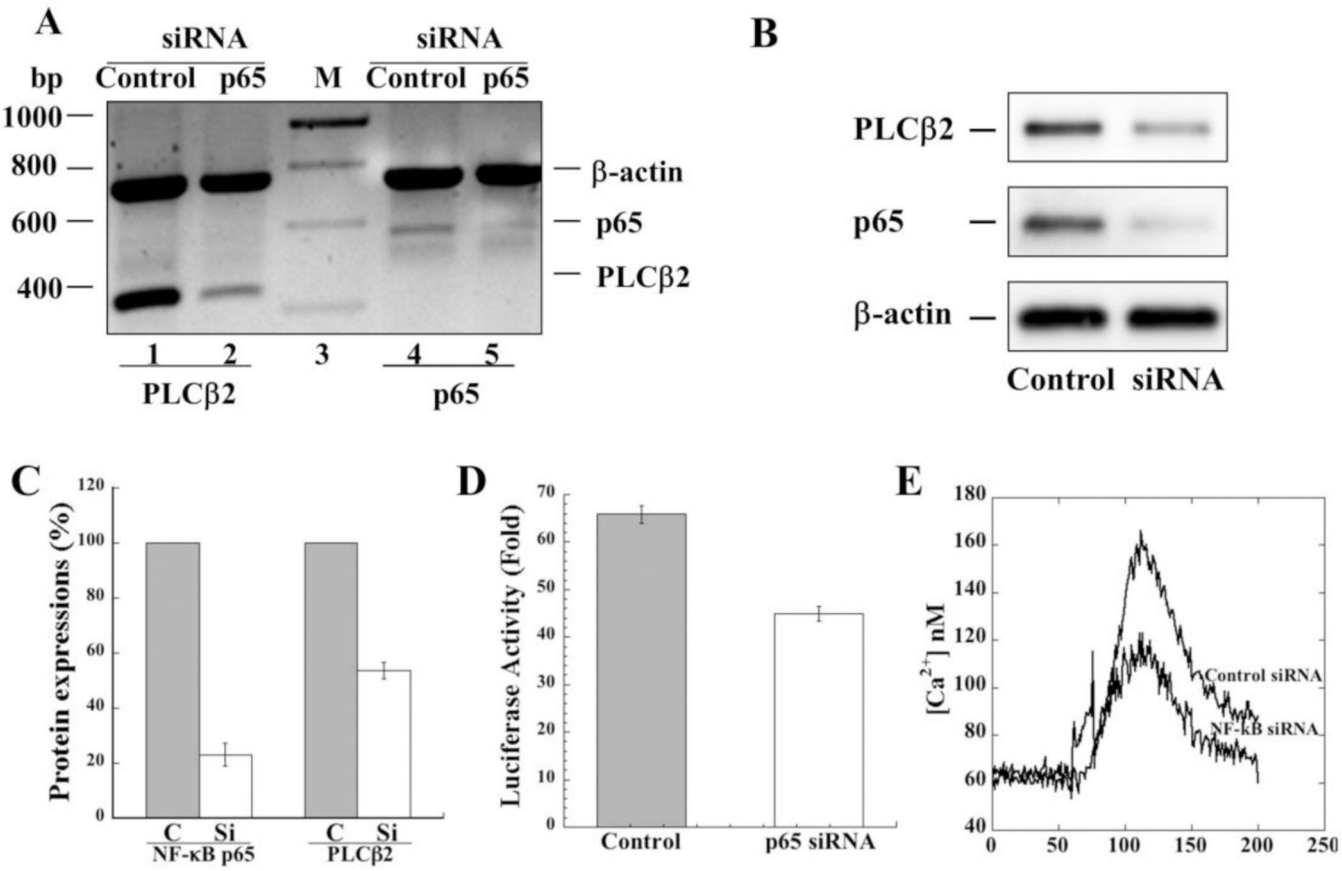

Figure 5. Effect of siRNA-mediated NF-kB p65 knockdown on PLC-β2 gene expression in HEL cells.

A. PLC-β2 (lanes 1,2) and NF-κB p65 mRNA (lanes 4, 5) levels were analyzed by RT-PCR 72 h after the cells were transfected with p65 siRNA or control siRNA. Also shown are actin mRNA as control in each lane. M, molecular markers. **B.** Immunoblotting of p65, PLC-β2 and actin. **C.** Shown mean of three immunoblotting experiments (mean ± S.D.). The ordinate shows NF-κB p65 or PLC-β2 protein relative to actin. **D.** Effect of p65 siRNA on *PLCB2* promoter activity at 48 h using *PLCB2* 5' construct −1791/−23. **E.** Effect on thrombin (1 U/ml)-induced rise in cytoplasmic Ca in HEL cells.

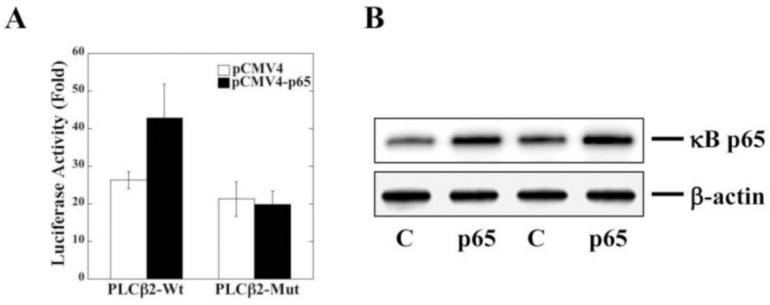

Figure 6. Effect of p65 overexpression on NF-kB protein and *PLCB2* promoter activity.

HEL cells were transfected with WT ***PLCB2*** promoter region (−1791/−23) containing p65 binding site (PLC-β2 Wt) or with 13 bp nt deletion (PLCβ2-mut) together with p65-pCMV4 expression plasmid or pCMV4 empty vector. **A.** Luciferase activity at 24 h showing increase in promoter activity with WT construct and loss of this increase with deletion of the NF-κB binding region. **B.** Western blot analysis of p65 and β-actin showing an increase with p65 overexpression compared to empty vector (C); each pair shown twice (representative of at least three experiments).

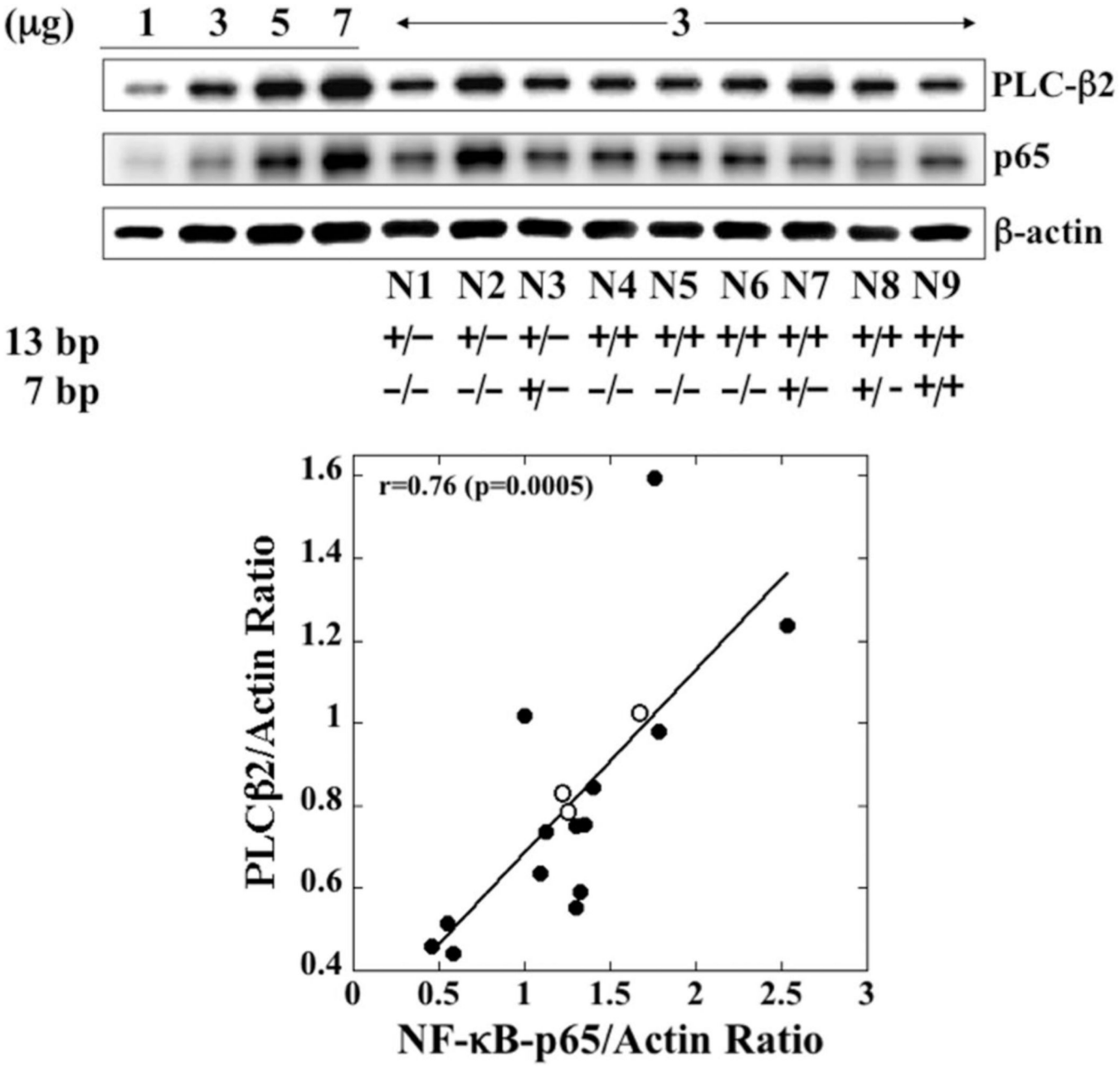

Figure 7. Platelet PLC-β2 and p65 NF-κB.

A. Immunoblots showing platelet PLC-β−2 p65 NF-κB and β-actin in 9 healthy subjects. The first 4 lanes show a standard curve (1, 3, 5, 7 μg protein). N1–9 are healthy subjects (3 μg). Also shown, genotype with respect to the presence (+) or absence (−) of the 13 bp region with NF-κB site and the 7 bp region with TFII-I site. **B.** Correlation of platelet PLC-β2 with NF-κB p65 protein in 17 subjects including 3 (open circles) with 13 bp deletion (both normalized against β-actin).

Table 1.

Platelet PLC-β2 and NF-κB p65 Levels by Genotype

Genotype				n	PLCβ2/Actin ratio	NF-κB/Actin ratio
					Mean ± SE	Mean ± SE
13 bp (−1645/−1633 nt)	+/+	All		14	0.82 ± 0.12	1.24 ± 0.21
		7bp	−/−	10	0.74 ± 0.076	1.32 ± 0.17
			+/−	3	1.02 ± 0.33	1.08 ± 0.38
			+/+	1	0.64	1.09
	+/−	All		3	0.88 ± 0.07	1.38 ± 0.14
		7bp	−/−	2	0.91 ± 0.12	1.46 ± 0.19
			+/−	1	0.84	1.23

PLC-β2 and NF-κB p65 levels were measured by immunoblotting in 17 control subjects.

(+) indicates wiltype sequence; (−) indicates deletion of the region.