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A novel function of Nur77: Physical and functional association with protein kinase C

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Abstract

Despite the involvement in diverse physiological process and pleiotropic expression profile, the molecular functions of Nur77 are not likely to be fully elucidated. From the effort to find a novel function of Nur77, we detected molecular interaction between Nur77 and PKC. Details of interaction revealed that C-terminal ligand binding domain (LBD) of Nur77 specifically interacted with highly conserved glycine-rich loop of PKC required for catalytic activity. This molecular interaction resulted in inhibition of catalytic activity of PKCθ by Nur77. C-terminal LBD of Nur77 is sufficient for inhibiting the phosphorylation of substrate by PKCθ. Ultimately, inhibition of catalytic activity by Nur77 is deeply associated with repression of PKC-mediated activation of AP-1 and NF-κB. Therefore, these findings demonstrate a novel function of Nur77 as a PKC inhibitor and give insights into molecular mechanisms of various Nur77-mediated physiological phenomena.

Keywords: Nur77; PKC; Ligand binding domain; Glycine-rich loop; Activation protein-1; Nuclear factor-κB

Nur77 (TR3/NGFI-B/N10/NAK/NR4A1), together with Nurr1 (NR4A2) and Nor-1 (NR4A3), belongs to NR4A subfamily of nuclear receptor superfamily [1]. Since its physiological ligand is not identified yet, Nur77 is classified as an orphan receptor. Nur77 is an immediate-early responsive gene induced by diverse stimuli including serum, growth factor, antigen receptor ligation, and apoptotic stimuli [2–4]. Similar to other nuclear receptors, Nur77 is composed of distinct structural domains, N-terminal activation function 1 (AF-1) domain required for recruiting transcriptional co-activators, DNA-binding domain with zinc-finger motif, and atypical C-terminal ligand binding domain without classical hydrophobic cleft for ligand binding [5,6]. As a monomeric or homodimeric form NR4A receptors bind to specific DNA sequences, termed NBRE or NurRE, in the promoter region of target genes [7,8].

Among pleiotropic physiological functions of Nur77, Nur77-mediated apoptosis has been extensively studied in T cells and several cancer cells [2,9–12]. In T cells, Nur77 is induced in CD4/CD8 double positive (DP) thymocytes during negative selection by TCR signal and plays an important role in TCR-mediated apoptosis in DP thymocytes and T cell hybridomas [2,9]. Up to date, two mechanisms have been proposed as mechanisms of Nur77-mediated apoptosis. As a transcription factor, Nur77 appears to upregulate apoptotic genes including FasL (Fas ligand), TRAIL (TNF-related apoptosis inducing ligand), NDG1 (Nur77 downstream gene 1), and NDG2

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[13,14]. Different from nuclear function, Nur77 also translocates to mitochondria, where it interacts with Bcl-2 and completely reverses the function of Bcl-2 from the anti-apoptotic to the pro-apoptotic through conformational change [11,12]. These different observations imply that Nur77 may have multiple functions related to apoptosis and prompted us to investigate a novel function of Nur77.

T cell receptor (TCR) engagement generates second messengers, including calcium and diacylglycerol (DAG). PKC is one of signal transducers downstream of calcium and DAG, playing a pivotal role in TCR-mediated T cell activation and IL-2 production [15]. PKC consists of a subfamily of at least 11 isotypes of serine/threonine kinases. Although their isotype-specific functions are not completely understood, multiple PKC isozymes are known to be expressed and to participate in the TCR-mediated responses such as proliferation, differentiation, and survival [16,17].

Here, we found PKCθ as a Nur77-interacting protein through yeast-two-hybrid method using cDNA library from Jurkat T cells [18] and Nur77-$^{\Delta}$DBD, known to be located in cytoplasm constitutively, as bait. Our data suggest that Nur77 is a physiological binding partner of PKC and regulates PKC activity via direct inhibition of catalytic activity, which is responsible for repression of TCR-mediated activation of AP-1 and NF-κB.

Materials and methods

Cell culture, antibodies, and reagents. Jurkat E6.1 (ATCC) and DO11.10 T cell hybridoma (a gift from Dr. Babara Osborne, University of Massachusetts, MA) were maintained in RPMI medium 1640 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, penicillin (50 μg/ml), and streptomycin (50 μg/ml). HEK293 were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (50 μg/ml), and streptomycin (50 μg/ml). Anti-myc (9E10) and anti-HA (16B12) antibodies were purchased from Covance, anti-flag M2 from Sigma, anti-PKCθ (clone 27), anti-Nur77 (clone 12.14), and anti-CD28 (CD28.2) from BD Pharmingen, anti-CD3 (OKT3) from Bioscience, and anti-PKCθ (H-7), anti-PKCα (E-7), anti-Nur77 (M-210), and rabbit IgG from Santa Cruz Biotechnology. HRP-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Pierce. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma. Human recombinant TNF-α was obtained from R&D systems.

Vector construction. Expression vectors encoding wild-type and truncated mutants of Nur77 were constructed by cloning the PCR products of Nur77 into pCS2+MT vector. To prepare recombinant Nur77 protein, PCR products of wild-type and truncated mutants were cloned into pGEX-5X-1 (Amersham–Pharmacia). PKCθ expressing pEF-neo-PKCθ wild-type, constitutively active mutant (A148E) and "kinase-dead" mutant (K409R) were kindly provided by Dr. Amnon Altman (The Burnham Institute, CA). Flag-tagged PKCθ wild-type and various truncated mutants were constructed by cloning the PCR products of PKCθ into pCDNA3.0-C-flag (Invitrogen). HA-tagged PKCα, PKCβ1, PKCδ, PKCε, PKCζ, PKCη, and PKCθ were provided by Dr. Soh (Inha University, Republic of Korea).

Transfection and co-immunoprecipitation. Jurkat T cells or DO11.10 cells were transiently transfected by electrophoration. Briefly, exponentially growing cells were harvested and washed with RPMI medium 1640 without serum. Cells (1×10^7) were then mixed with indicated constructs and transferred to a Gene Pulser Cuvette (Bio-Rad). Electroporation was performed at 250 V and 975 μF using a Gene Pulser II (Bio-Rad). For co-immunoprecipitation assays, HEK293 cells were transfected with various constructs by using the calcium phosphate method. After an overnight incubation, cell lysates were prepared using lysis buffer (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, and 0.5% NP-40) and then immunoprecipitated with appropriate antibodies. Precipitates were then subjected to SDS–PAGE and immunoblotted with indicated antibodies. For analyzing endogenous interaction, DO11.10 cells were stimulated with PMA/ionomycin (25 ng/ml, 0.5 μM) for 3 h. Cell lysates were prepared and then pre-cleared by incubating with protein A-coated agarose (Santa Cruz Biotechnology) for 4 h. Pre-cleared cell lysates were incubated with rabbit IgG or anti-Nur77 (M-210) antibody for 4 h and for an additional 2 h in the presence of protein-A coated agarose. Samples were subjected to immunoblotting using anti-PKCθ (E-7) or anti-Nur77 (12.14).

In vitro translation, GST-pull-down, and far-Western blotting. pcDNA3.0-flag-PKCθ full-length and various deletion mutants were transcribed and translated *in vitro* using the TNT T7 system (Promega) in the presence of [^{35}S]methionine (Amersham–Pharmacia) according to manufacturer's instructions. For GST-pull-down assays, GST-fused Nur77 was mixed with 40 μl of [^{35}S]-labeled PKCθ for 2 h at 4 °C. Samples were subjected to SDS–PAGE, dried, and processed for autoradiography. For far-Western blotting, GST and GST-Nur77 recombinant proteins were subjected to SDS–PAGE and blotted onto nitrocellulose membrane, which were exposed with 80 μl [^{35}S]-labeled PKCθ for 2 h at 4 °C. After extensive washing, bands were visualized by autoradiography.

In vitro kinase analysis. Wild-type or active mutant PKCθ was prepared from HEK293 cells transfected with corresponding plasmids. The immunoprecipitates were resuspended in 20 μl of kinase reaction buffer (20 mM Hepes at pH 7.5, 10 mM $MgCl_2$, and 0.1 mM EGTA) containing myelin basic protein (MBP, 1 μg) and [γ-^{32}P]ATP (5 μCi) in the presence or absence of GST, GST-Nur77, or GST-Nur77-truncated protein for 30 min at 30 °C. Reactions were stopped by mixing with 5× SDS–substrate buffer and subsequent boiling for 5 min. Prepared samples were subjected to SDS–PAGE and autoradiography.

Reporter gene analysis. Reporter analysis was performed using a luciferase assay kit (Tropix). Briefly, Jurkat T cells were electroporated with reporter vectors driven by AP-1 or NF-κB element along with indicated constructs. After 10 h of incubation, cells were stimulated with anti-CD3/CD28 antibody (10, 5 μg/ml), PMA/ionomycin (25 ng/ml, 0.5 μM), or TNF-α (20 ng/ml) for 4 h. Cell lysates were obtained using lysis buffer, and luciferase activity determined according to the manufacturer's instructions. Protein concentrations of cell lysate were measured using BCA protein assay reagent (Pierce). Luciferase activity was normalized versus protein concentration. Relative luciferase activity (RLA) was calculated as a percentage of that of the positive control group.

Statistical analysis. The significance of differences between experimental conditions was determined by Student's *t* test. Data were presented as means ± SD and statistical significance was indicated by two-tailed *P* value.

Results

Physical interaction between Nur77 and PKC

We first reconfirmed the interaction between Nur77 and PKCθ. When myc-tagged Nur77, flag-tagged PKCθ or both were expressed in HEK293 cells and applied to co-immunoprecipitation assay, we found that Nur77 or PKCθ was specifically immunoprecipitated with PKCθ or Nur77 reciprocally (Fig. 1A). To further confirm the interaction between Nur77 and PKCθ, we applied *in vitro* binding assay. GST-pull-down assay and far-Western blotting revealed that *in vitro* translated-PKCθ specifically interacted with GST-fused Nur77, not with GST control protein

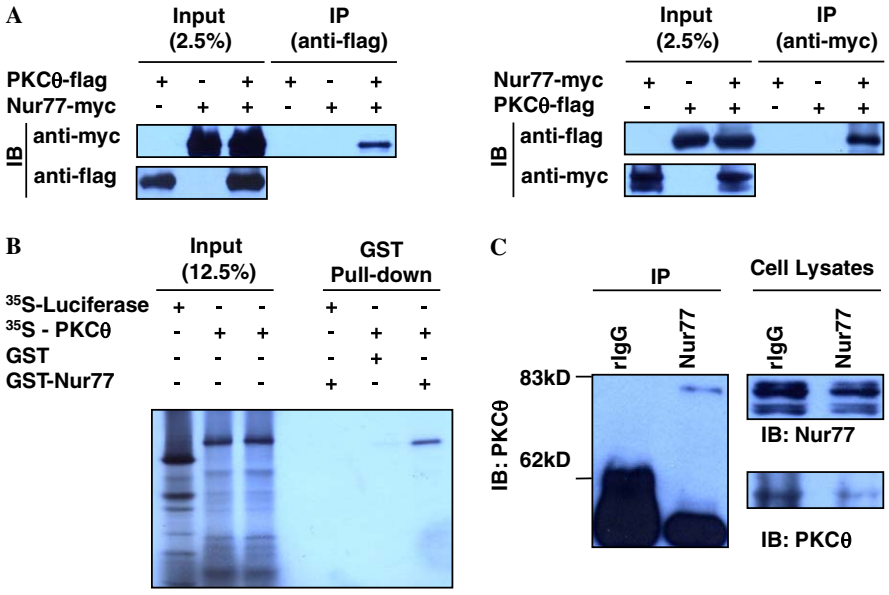

Fig. 1. Nur77 physically associates with PKCθ. (A) Co-immunoprecipitation of Nur77 with PKCθ. Myc-tagged Nur77 and/or flag-tagged PKCθ were expressed in HEK293 cells. Cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with the indicated antibodies. (B) [^{35}S]-labeled PKCθ was subjected to GST-pull-down assay with GST or GST-Nur77. (C) Cell lysates prepared from PMA/ionomycin-treated DO11.10 cells were immunoprecipitated and immunoblotted with the indicated antibodies.

(Fig. 1B and see Supplementary material). We next examined whether this molecular interaction would occur in endogenous expression level of two molecules. To this end, we used DO11.10 T cell hybridoma because it was known to express the higher level of Nur77 by CD3/CD28 or PMA/ionomycin treatment [3,9]. The lysate obtained from PMA/ionomycin-treated DO11.10 cells was immunoprecipitated with rabbit IgG control or anti-Nur77 antibodies. As shown in Fig. 1C, PKCθ was specifically immunoprecipitated with anti-Nur77 antibody, not with control rabbit IgG, indicating the interaction of Nur77 with PKCθ under the physiological condition. From these results, we concluded that PKCθ was a physiological interacting partner of Nur77.

Next, we tested whether Nur77 would interact with other isotypes of PKC, because 11 isotypes of PKC contain highly conserved motives in their sequences [19]. In co-immunoprecipitation assays, we observed that Nur77 interacted with all PKC isotypes tested (see Supplementary material). Therefore, this finding suggested that Nur77 interacted with conserved motif of PKCs and might have a common role via physical association with multiple PKC isotypes.

Interaction between ligand binding domain of Nur77 and catalytic domain of PKCθ

To elucidate the detailed molecular interaction between Nur77 and PKCθ, we sought the exact motif responsible for interaction. Nur77 is composed of three functional domains, N-terminal transactivation domain (TAD/AF-1), central DNA binding domain (DBD), and C-terminal ligand binding domain (LBD). Therefore, we constructed myc-tagged TAD, LBD, and Nur77-ΔDBD, and expressed in HEK293 cells along with PKCθ wild-type. By co-immunoprecipitation assay, we found that LBD of Nur77 was sufficient for interaction with PKCθ (Fig. 2A). Next, we investigated the exact binding motif of PKCθ to Nur77. A series of truncated PKCθ-mutants were constructed and expressed in HEK293 cells along with Nur77 wild-type. Co-immunoprecipitation assay showed that Nur77 interacted with C-terminal catalytic domain (CD) of PKCθ, not with N-terminal regulatory domain (RD) (Fig. 2B, lane 3). Furthermore, deletion of glycine-rich loop (ATP-binding motif) from catalytic domain (CD^{Δ} ATP and CD^{Δ} HD^{Δ} ATP) completely abolished the interaction with Nur77 (Fig. 2B, lanes 4 and 6), indicating that region containing glycine-rich loop was essential for interaction with Nur77. Because LBD was interacting motif of Nur77 with PKCθ, we further tested whether LBD would interact with glycine-rich loop. As wild-type Nur77, LBD interacted only with catalytic domain of PKCθ containing glycine-rich loop in co-immunoprecipitation analysis (see Supplementary material) and in GST-pull-down assay using GST-fused LBD and in vitro-translated PKCθ (data not shown). Because glycine-rich loop was one of highly conserved motives of PKC and we found that Nur77 interacted with other PKC isotypes, we examined the interaction between LBD and other PKC isotypes (i.e., PKCα, PKCδ, and PKCζ) [19]. As expected, LBD interacted with all PKC isotypes tested (see Supplementary material), suggesting that Nur77 interacted with PKC through a conserved region containing glycine-rich loop.

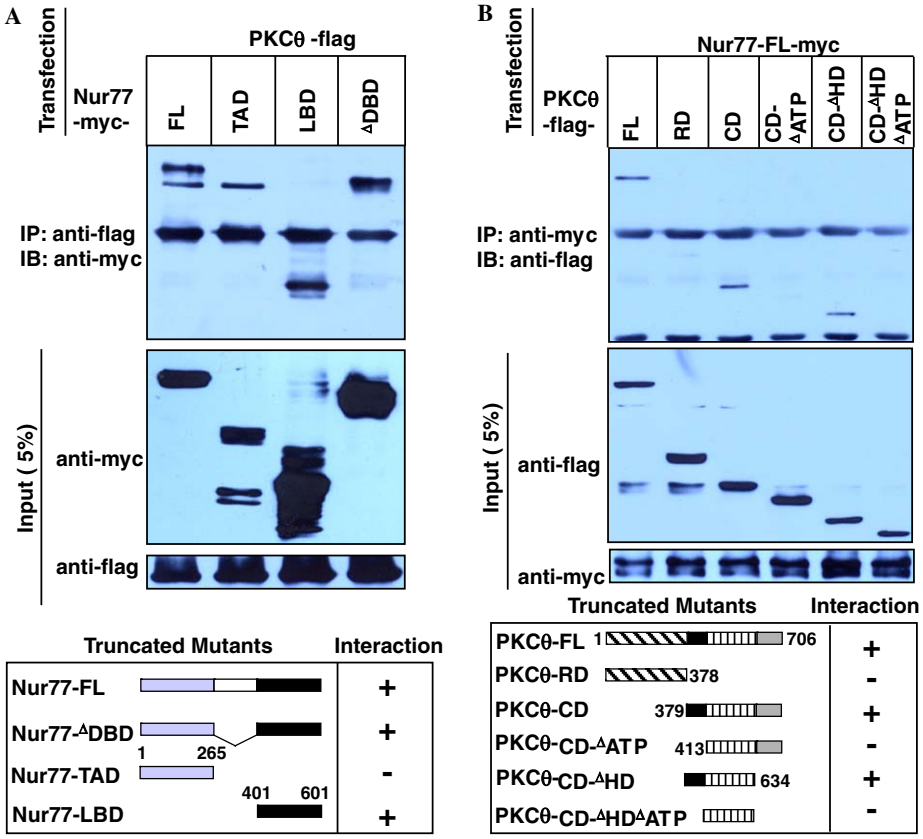

Fig. 2. LBD of Nur77 binds to the glycine-rich loop of PKCθ. (A) Indicated constructs of Nur77 were expressed in HEK293 cells. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (B) Using truncated mutants of PKCθ (FL, full-length; RD, regulatory domain; CD, catalytic domain; CD-$^{\Delta}$ATP, catalytic domain without glycine-rich domain; CD$^{\Delta}$HD, catalytic domain without hydrophobic domain and CD$^{\Delta}$HD$^{\Delta}$ATP) co-immunoprecipitation was performed as described above.

Inhibition of kinase activity by Nur77

The activation of PKCs is regulated by opening a catalytic cleft through stimulator-induced conformational changes and by the phosphorylation of specific sites by upstream kinases and by autophosphorylation [20]. Since glycine-rich loop of PKC was a motif required for binding of ATP, one co-substrate of PKC, and structurally close to catalytic cleft and activation loop of PKC, we speculated that the binding of Nur77 to the glycine-rich loop of PKC might change the catalytic activity of PKC [20,21]. We thus investigated whether Nur77 would inhibit catalytic activity of PKCθ using an *in vitro* kinase (IVK) assay. When GST-fused Nur77 was incubated with immunoprecipitated active PKCθ, it significantly reduced PKCθ-driven phosphorylation of myelin basic protein (MBP) and the autophosphorylation of PKCθ (Fig. 3A and B). GST-Nur77 also inhibited the phosphorylation of MBP by the PMA-activated wild-type of PKCθ (data not shown). Since Nur77 inhibited catalytic activity of PKCθ, our next question was which region of Nur77 would be responsible for inhibiting PKC activity. To address this question, we prepared a series of truncated proteins of Nur77 and applied them to kinase assay. Result showed that GST-fused Nur77 wild-type (GST-FL) and the LBD (GST-LBD) almost completely inhibited the phosphorylation of MBP by PKCθ (Fig. 3C), which is consistent with the binding assay result, i.e., that LBD specifically interacted with glycine-rich loop of PKCθ (see Supplementary material). Because large amounts of one substrate (Nur77) may compete with other substrates (MBP) for limited amount of enzyme (PKCθ), we tested the possibility that Nur77 might be a direct substrate of PKCθ. However, GST-fused wild-type Nur77 (M.W., 100 kDa) and LBD (M.W., 50 kDa) were not phosphorylated by PKCθ active mutant *in vitro* kinase assay (Fig. 3B, data not shown), indicating that LBD was not a substrate of PKCθ.

Repression of PKC-mediated transactivation of AP-1 and NF-κB by Nur77

Above data of physical interaction between Nur77 and PKCs and functional inhibition of catalytic activity strongly suggested the repressive function of Nur77 in PKC-mediated responses. Therefore, next question was what would be consequences of this interaction. Since PKCθ was known to be involved in TCR-mediated activation of activation protein-1 (AP-1) and nuclear factor-κB (NF-κB) [22], we

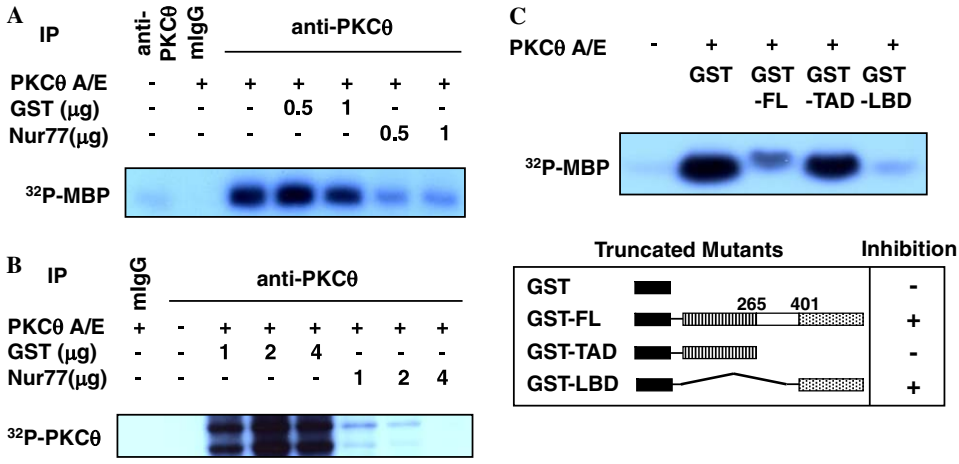

Fig. 3. Nur77 inhibits catalytic activity of PKCθ. Immune complexes of constitutively active PKCθ (PKCθ A/E) were prepared from cell lysate of HEK293 cells expressing active PKCθ. IVK (*in vitro* kinase) assay was performed in the presence of GST, or GST-Nur77 proteins. [^{32}P]-MBP (A) or autophosphorylated PKCθ (B) was visualized by autoradiography. (C) Using active PKCθ immune complex, IVK assay was done in the presence of GST control or GST-fused truncated proteins of Nur77.

examined the effect of Nur77 on PKCθ-driven activation of AP-1 and NF-κB. To this end, Jurkat leukemic T cells were transfected with AP-1 or NF-κB-driven reporter plasmids along with or without increasing amount of Nur77 and then stimulated with anti-CD3/CD28 or PMA/ionomycin. Reporter analysis revealed that Nur77 obviously repressed TCR signaling-mediated activation of AP-1 and NF-κB (Fig. 4A). To examine whether Nur77-mediated repressive activity is specific to PKCθ, we tested the effect of Nur77 on TNF-α (tumor necrosis factor α)-mediated NF-κB activation. As shown in Fig. 4B, Nur77 did not repress TNF-α-mediated NF-κB activation, indicating that the repressive activity of Nur77 is specific to a TCR-mediated pathway involving PKCθ as a signaling mediator. To reconfirm PKC-specific repressive activity of Nur77, we performed reporter analysis using PKCθ or PKCα active mutants. As

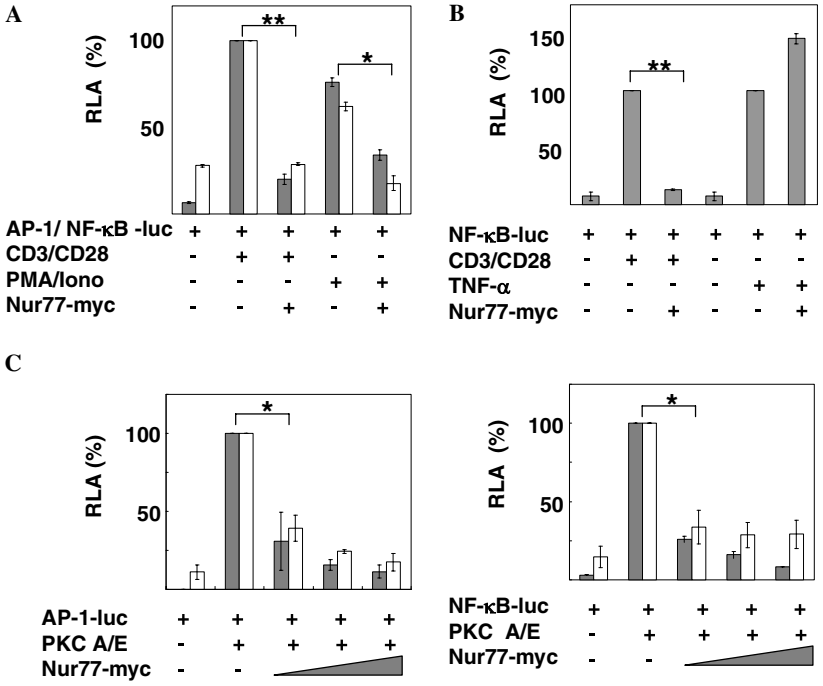

Fig. 4. Nur77 represses PKC-mediated activation of AP-1 and NF-κB. (A) Jurkat T cells transfected with the indicated vectors were stimulated with anti-CD3/CD28 or PMA/ionomycin. Luciferase activity of AP-1 (grey bars) or NF-κB (blank bars) reporter plasmid was measured as described in Materials and methods. (B) Jurkat T cells transfected with indicated constructs were stimulated with anti-CD3/CD28 or TNF-α. (C) Jurkat T cells were transfected with reporter plasmid, active PKCθ (grey bars), active PKCα (blank bars), and increasing amount of Nur77 (1, 2, and 4 μg, respectively). After 10hr of incubation, luciferase activity was measured. All data were presented as mean values of relative luciferase activity (RLA) ± SD ($n = 3$, two tailed $^{*}P < 0.05$, $^{**}P < 0.001$).

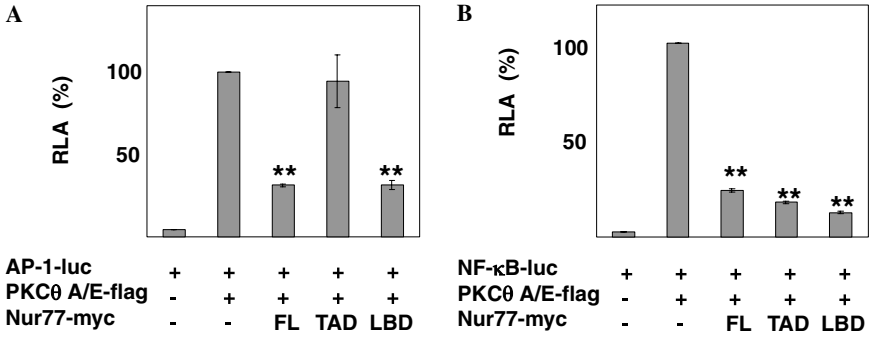

Fig. 5. LBD represses PKCθ-driven activation of AP-1 (A) and NF-κB (B). Jurkat T cells were transfected with indicated plasmids. Luciferase activity was measured after 10 h or incubation. Data were presented as mean values of RLA ± SD ($n = 3$, two tailed $^{**}P < 0.001$ as compared to control group without Nur77).

expected, Nur77 repressed both the transactivations of AP-1 and NF-κB driven by active mutants of PKCθ or, to a lesser extent, PKCα in Jurkat leukemic T cells and in other cell types (Fig. 4C and data not shown).

Next, we asked whether LBD could repress PKCθ-mediated activation of AP-1 and NF-κB. To address this question, Jurkat cells were transfected with Nur77 deletion mutants along with AP-1 or NF-κB reporter gene. In reporter analyses, wild-type (FL) and LBD of Nur77 significantly repressed PKCθ-mediated activation of AP-1 and NF-κB reporter genes (Fig. 5A and B). However, unexpectedly, N-terminal TAD also repressed PKCθ-mediated NF-κB activation without repression of AP-1 activation, suggesting that repressive activity of LBD was associated with molecular interaction with PKCθ, whereas that of TAD was not dependent on interaction with PKCθ and specific to NF-κB pathway (Figs. 2 and 5A and B). Summarizing, LBD of Nur77 represses PKCθ-mediated activation of two major transcription factors of T cell, which is consistent with results of mapping and kinase assays (Figs. 2A and 3C).

Discussion

In this study, we demonstrate a novel Nur77-interacting partner, PKC, and a novel function of Nur77 as a PKC inhibitor. Nur77 is inducible immediate-early gene and involved in wide range of physiological events including apoptosis, steroidogenesis, and proliferation [23]. Although most of cellular functions of Nur77 were dependent on transactivation activity, recent reports suggested new paradigm of its function as a regulator of another protein through protein–protein interaction [12].

Although Nur77 has been demonstrated to be involved in apoptosis of T cells and several cancer cells and inhibition of NF-κB, it has also been suggested that Nur77 is a survival factor during TNF receptor signaling and inhibits apoptosis through enhancing NF-κB activity [3,24–26]. This paradoxical discrepancy of Nur77 function plausibly implies that its function may be different in a cellular or signal context dependent manner. Although both of TCR and TNFR (TNF receptor) signaling pathways are converged on NF-κB activation, membrane proximal events are different from each other. TCR-mediated NF-κB activation is mediated by recruitment of PKC to membrane signaling complex. Therefore, our data, molecular interaction between Nur77 and PKC and specific repression of PKC-mediated NF-κB activation, can be plausible explanation for previously reported functional discrepancy of Nur77 in a signal context dependent manner.

Mapping results indicated that LBD of Nur77 interacted with and inhibited PKC. Consistent with these results, LBD functionally repressed PKC-mediated activation of AP-1 as well as NF-κB. However, N-terminal TAD also repressed PKC-mediated NF-κB activation without repression of AP-1 activation. Harnat et al. demonstrated TAD-dependent repression of NF-κB activation and hypothesized that Nur77, like glucocorticoid receptor, represses NF-κB activity through the direct molecular interaction of transcription factors [24]. Although further studies are required, our results demonstrate that repression of NF-κB activity by TAD is not likely to be direct modulation of PKC or upstream signaling components because TAD does not interact with PKC nor inhibit PKC activity. Taken together, previous reports and our results suggest multiple mechanisms of NF-κB modulation by Nur77.

Similar to binding of Nur77 to glycine-rich loop (ATP-binding motif) of PKC, p16INK4a, a CDK inhibitor, binds next to the ATP-binding site of CDK6 and, ultimately leads to the distortion of catalytic cleft and inhibition of ATP binding to CDK6 [27]. Furthermore, p16INK4a also binds to glycine-rich loop region of JNK3 and inhibits c-Jun phosphorylation by JNK3 [28], suggesting that binding of inhibitor to the highly conserved glycine-rich-loop of kinases is one of common molecular mechanisms of inhibition. Although further structural investigation is required, it is plausible that Nur77 binds to glycine-rich-loop of PKC and inhibits PKC activity in a manner similar to that of p16INK4a.

Although details of physiological consequences of cross-talk between Nur77 and PKC require further studies, our results are consistent with the previous findings that Nur77 has a pro-apoptotic function and suggest the possibility that the blockade of PKC function by Nur77 may be a prerequisite of apoptosis, because PKC-mediated NF-κB

activation, which ultimately leads to expression of anti-apoptotic genes, and PKC-mediated modulation of pro-apoptotic molecule are known to be associated with protection of cells from apoptotic signals [29,30].

In summary, we have demonstrated that physical and functional cross-talk between Nur77 and PKC. From this study, we suggest a novel function of Nur77 and provide molecular basis of Nur77-mediated apoptosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.07.167.

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