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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 ($1 \times 10^7$) 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