EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 37, No. 5, 466-475, October 2005

Menin represses JunD transcriptional activity in protein kinase Cθ-mediated Nur77 expression

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Accepted 9 September 2005

Abbreviations: bis-IM, bisindoylmaleimide; HDAC, histone deacetylase; MEF2, myocyte enhancing factor 2; MITR, MEF2-interacting transcription repressor; PKC, protein kinase C; RT, reverse transcription; TCR, T cell receptor

Abstract

TCR signaling leading to thymocyte apoptosis is mediated through the expression of the Nur77 family of orphan nuclear receptors. It has been shown that the Nur77 promoter is activated by at least two signaling pathways, one mediated by calcium and the other by protein kinase C (PKC). MEF2D has been known to regulate Nur77 expression in a calcium-dependent manner. The mechanism by which calcium regulates MEF2D is through dissociation of calcium-sensitive MEF2 corepressors (Cabin1/HDACs, HDAC4/5) and the association with calcineurin-activated transcription factor NF-AT and the coactivator p300. However, little is known about how PKC activates the Nur77 promoter. Herein, we report that PKCθ targets AP-1 like response element in the Nur77 promoter where JunD constitutively binds. PKCθ triggers mitogen-activated protein kinase-inediated phosphorylation of JunD, and increases transcriptional activity of JunD, cooperatively with p300. Menin is identified as the transcriptional corepressor for JunD via recruitment of mSin3-istone deacetylases. In fact, Menin represses PKCθ/p300-mediated transcriptional activity of JunD in T cell. Its dynamic regulation of histone modifiers with JunD is responsible for PKCθ-synergistic effect on Nur77 expression in T cell.

Keywords: histone deacetylase; protein kinase C; transcription factors; transcription factor AP-1

Introduction

T cell receptor (TCR)-mediated apoptosis of immature thymocytes is important for eliminating self-reactive T cells. The Nur77 family of transcription factors has been identified as crucial mediators for TCR-mediated thymocyte apoptosis (Sebzda *et al.,* 1999; Winoto and Littman, 2002). Nur77 (NGFI-B/TR3) is an NR4A1-3 subgroup of orphan nuclear receptors (Nur77, NOR- 1, Nurr1) and mediates apoptotic pathways in prostate cancer cells, lung cancer cells, gastric cancer cells as well as immature T cells (Liu *et al.,* 1994; Woronicz *et al.,* 1994; Weih *et al.,* 1996, Uemura and Chang *et al.,* 1998; Li *et al.,* 2000; Lu *et al.,* 2002).

Recent years have witnessed significant advances in our understanding of the calcium-dependent intracellular signaling pathway from TCR to Nur77 transcription. Myocyte enhancing factor 2 (MEF2) has been identified as a key calcium-dependent transcription factor that regulates gene expression in muscle, neuronal, and immune cells (McKinsey *et al.,* 2002). MEF2D, a predominant isoform in T cells, is found to bind to two calcium-dependent DNA elements in the Nur77 promoter and to mediate calcium-dependent induction of Nur77 (Woronicz *et al.,* 1995). We have previously shown that two distinct calcium-mediated pathways are involved in the activation of MEF2 transcription: (i) Calcium, calmodulin-dependent dissociation of a family of MEF2 transcription repressors. Three distinct types of repressors for MEF2 have been identified, Cabin1/Cain (Lai *et al.,* 1998; Sun *et al.,* 1998; Youn *et al.,* 1999; Youn and Liu, 2000), HDAC4, 5, 7, 9 (Miska *et al.,* 1999; Wang *et al.,* 1999; Dressel *et al.,* 2001), and MITR (Sparrow *et al.,* 1999). Among these corepressors, Cabin1 is capable of recruiting Class I HDACs via binding to the corepressor mSin3 while MITR can directly recruit Class I HDACs. It

was recently reported that HDAC7 serves as a thymus-specific class II histone deacetylase, and regulates Nur77 transcription and TCR-mediated apoptosis (Dequiedt *et al.,* 2003). In the absence of an appropriate TCR signal, MEF2 is bound to most, if not all, of these functionally redundant repressors, silencing the Nur77 promoter. Upon TCR activation and the accompanying increase in intracellular calcium concentration, activated calmodulin binds to all repressors in a manner that is competitive against MEF2, releasing the repressors from MEF2 and allowing the binding of p300 (Youn and Liu, 2000; Youn *et al.,* 2000b). Moreover, calmodulin-dependent kinase (CaMK) I and IV also appear capable of dissociating HDAC4 and 5 from MEF2, in part by causing nuclear export of HDAC4/5 (McKinsey *et al.,* 2000). (ii) Binding of calcineurin-activated NF-AT to MEF2D. NF-AT, a well-known calcineurin substrate in non-activated T cells, translocates from the cytosol into the nucleus where it forms a ternary complex with MEF2 and p300, leading to full activation of Nur77 expression (Blaeser *et al.,* 2000; Youn *et al.,* 2000a).

In addition to calcium signal, TCR also activates protein kinase C (PKC), which acts synergistically with calcium signal to activate the Nur77 promoter and thymocyte apoptosis. PKC family of serine/threonine kinases is thought to play roles in a variety of tissues and cell types (Altman *et al.,* 2000; Seo *et al.,* 2004). Among these, PKCθ is expressed predominantly in skeletal muscle and lymphoid organs (Osada *et al.,* 1992; Baier *et al.,* 1993). PKCθ has been known to regulate the transcription of interleukin-2 in T lymphocytes primarily through the activation of NF-κB (Sun *et al.,* 2000). The transcription factor mediating the activation of Nur77 by PKCθ, however, remains unknown. Menin is a tumor suppressor protein encoded by MEN1 (multiple endocrine neoplasia type I), a causative gene associated with tumors of parathyroid, enteropancreatic neuroendocrine tissue and anterior pituitary (Chandrasekharappa *et al.,* 1997). Menin has been shown specifically interacts with JunD but not with other members of Jun family (c-Jun, JunB), as their interactions are mediated through the far N-terminal region of JunD that is missing in c-Jun and JunB (Aggarwal *et al.,* 1999). In addition to JunD, Menin is also known to interact with other transcription factors, including NF-κB, Smad3, and Pem, implicating a general role of Menin in regulating transcription (Heppner *et al.,* 2001; Kaji *et al.,* 2001; Lemmens *et al.,* 2001). Menin is known to repress JunD-activated transcription. The detailed molecular mechanism by which Menin represses JunD, however, is still unclear. Gobl *et al.,* recently showed that HDAC inhibitor, trichostatinA reverses Menin-mediated JunD transcriptional repression, suggesting that HDACs may be involved in Menin-mediated transcriptional repression of JunD and other transcription factors (Gobl *et al.,* 1999). We recently found that Menin represses JunD transcriptional activity by recruiting an mSin3A-histone deacetylase complex (Kim *et al.,* 2003).

In this study, we identify the primary PKCθ-responsive element in the Nur77 promoter as that of AP-1 and show that JunD is the predominant isoform of AP1 that is bound to the site on the Nur77 promoter. We demonstrated that PKCθ activates JunD transcriptional activity through MAP kinase-mediated phosphorylation of JunD. We previously showed that Menin represses JunD transcriptional activity through recruitment of mSin3-histone deacetylase complexes (Kim *et al.,* 2003). The Menin-mediated JunD transcriptional repression is relieved by PKCθ/p300-mediated transactivation of JunD. Taken together with our previous results that calcium regulates MEF2 transcriptional activity by controlling association of MEF2 with Cabin1-HDAC complexes and p300 acetyltransferase during T cell apoptosis (Youn *et al.,* 1999; 2000a; Youn and Liu, 2000), these results suggest another regulatory mechanism by which PKCθ synergizes with calcium to activate Nur77 expression by controlling the association of JunD with HDAC and HAT-containing complexes.

Materials and Methods

Cell culture and transfections

Jurkat T cells and DO11.10 T hybridoma cells were maintained in RPMI 1640 media containing 1 mM glutamine, 10% fetal bovine serum, and 50 U/ml of streptomycin and penicillin. 293 human embryonic kidney (HEK293) cells were grown in DMEM media containing 10% fetal bovine serum and 50 U/ml of streptomycin and penicillin. T cells were transfected by electrophoration (250V, 950μF). HEK293 cells were transfected with lipofectamine reagent per manufacturer's protocol (Invitrogen).

DNA constructions

The constructs for Nur77-promoter driven reporter genes used in this study were prepared by inserting the PCR fragments of Nur77 promoter into PGL2-basic vector or pGL2-SV40 promoter vector (Promega). PKCθ constructs [pEFPKCθ (A148/E) and pEFPKCθ (K409/R)] were kindly provided by Dr. A. Altman (La Jolla Institute for Allergey and Immunology, San Diego), Menin construct by Dr. C. X. Zhang (CNRS-UMR, France) and Dr. S. K. Agarwal (NIH, Bethesda), pGEX-junD (1-347) by Dr. A. Gobl (Uppsala University Hospital, Sweden). Deletion mu-

tants of Menin were made by subcloning of PCR fragments into pcDNA3-myc/His vector (Invitrogen). For making mammalian expression vector for HA-tagged human JunD, PCR fragment of JunD (1-347) was inserted into pSG5-HA vector. HA-tagged PICOT expression vector was prepared by inserting cDNA of human PICOT obtained from Marathon-ready human lymph node library (Clontech) into pSG5-HA vector.

Gel shift assay and biotin-streptoavidin affinity pull-down assay

The nuclear extracts from PMA/ionomycin treated DO11.10 cells were prepared by method as described previously (Sun *et al.,* 1998). The nuclear extracts were incubated with c-jun, JunD antibodies (Santa Cruz) for 30 min, and incubated with ^{32}P-labelled Nur77 promoter probe for further 30 min, and run at 200 V on 4% polyacrylamide gel (sense primer, 5'-GCTCCCTGCGTCAATGGAACC-3'; antisense primer, 5'-GGTTCCATTGACGCAGGGAGC-3'). To confirm the binding of JunD to Nur77 promoter, biotin-labelled Nur77 oligonucleotides (0.1 μM) were incubated with DO11.10 cell nuclear extracts and streptavidin agarose (Oncogene) in a buffer [25 mM HEPES (pH 7.4), 5% glycerol, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT and 1mM PMSF] for 2h, washed three times with washing buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 20% glycerol, 0.1% NP-40], subjected to 12% SDS-PAGE and transferred onto NC membrane, and probed with anti-JunD antibody.

Coimmunoprecipitation

Transiently transfected DO11.10 T cells or HEK293 cells were harvested after 24 h and lysed with a lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 1 mM PMSF]. Cell lysates were immunoprecipitated with suitable antibodies along with protein-A/G beads (Santa Cruz). Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with appropriate antibodies. Antibodies for anti-Menin, anti-Nur77, anti-mSin3A were purchased from Santa Cruz Biotechnology, anti-c-myc and anti-HA monoclonal antibodies from Covance and anti-flag (M2) monoclonal antibody from Sigma.

RNA extraction and reverse transcription (RT)-PCR analysis

Total RNAs were extracted from murine DO.11.10 cells with TRIzol reagent (Life Technologies, Inc.). cDNAs were synthesized using reverse transcriptase cDNA synthesis kit (Takara, Japan) with total RNAs as template. The PCR primers used in this study were as follows: Nur77, 5'-GTTGATGTTCCC-GCCTTTGCC-3' and 5'-TCAGAAAGACAATGTGT-CCAT-3'; β-actin, 5'-TGACCCAGATCATGTTTGAG-ACC and 5'-CCATACCCAAGAAGGAAGGC-3'.

Phosphorylation of JunD

PKCθ (A148/E)-transfected cell lysates were immunoprecipitated with either anti-PKCθ (Transduction laboratories), anti-JNK, or anti-ERK antibodies (Santa Cruz). Immunoprecipitates were incubated with 1 μg of purified recombinant GST-JunD in kinase reaction buffer for 30 min, then reactants were subjected to 10% SDS-PAGE. Phosphorylation of GST-JunD was detected by anti-phospho-junD (Ser100) antibody (Upstate).

Results

PKC is involved in Nur77 induction in T cells

To examine the role of PKC in Nur77 induction during T cell apoptosis, we first investigated the effect of PMA, along with ionomycin or both, on Nur77 expression at the mRNA level. When PMA (40 μM) was treated for 1 h, mRNA of Nur77 was induced albeit lower to the treatment of both PMA and ionomycin (Figure 1A). To determine the effect of PMA on the expression of Nur77 at the protein level, we treated DO.11.10 cells with PMA, ionomycin or both for 3 h. Interestingly, PMA could not trigger the expression of Nur77 protein, whereas ionomycin could result in the expression of Nur77 protein. In fact, we observed that PMA transiently induced mRNA of Nur77 within 1 h, thereafter, mRNA of Nur77 disappeared significantly (data not shown), indicating that PMA alone cannot prolong the expression of Nur77. Nonetheless, PMA synergistically affected the induction of Nur77 protein along with inomycin (Figure 1B), implying that PMA, at least, partially contributes to the induction of Nur77. The optimal induction of Nur77 is achieved with a combination of PMA and ionomycin (Figure 1A, B), indicating that PMA signal is likely to be involved in the activation of the Nur77 promoter. To confirm the participation of PKC, we pretreated DO11.10 cells with bisindolylmaleimide-I (bis-IM) for 30 min followed by the activation of DO11.10 cells with PMA and ionomycin for another 3 h. Bis-IM inhibited Nur77 induction in a dose-dependent manner (Figure 1C). The significant, albeit incomplete, inhibition of Nur77 by bis-IM supports that PKC is involved in Nur77 induction. It has been known that PKCθ is a predominant PKC isoform for T cell development and/or activation (Altman *et al.,* 2000). To

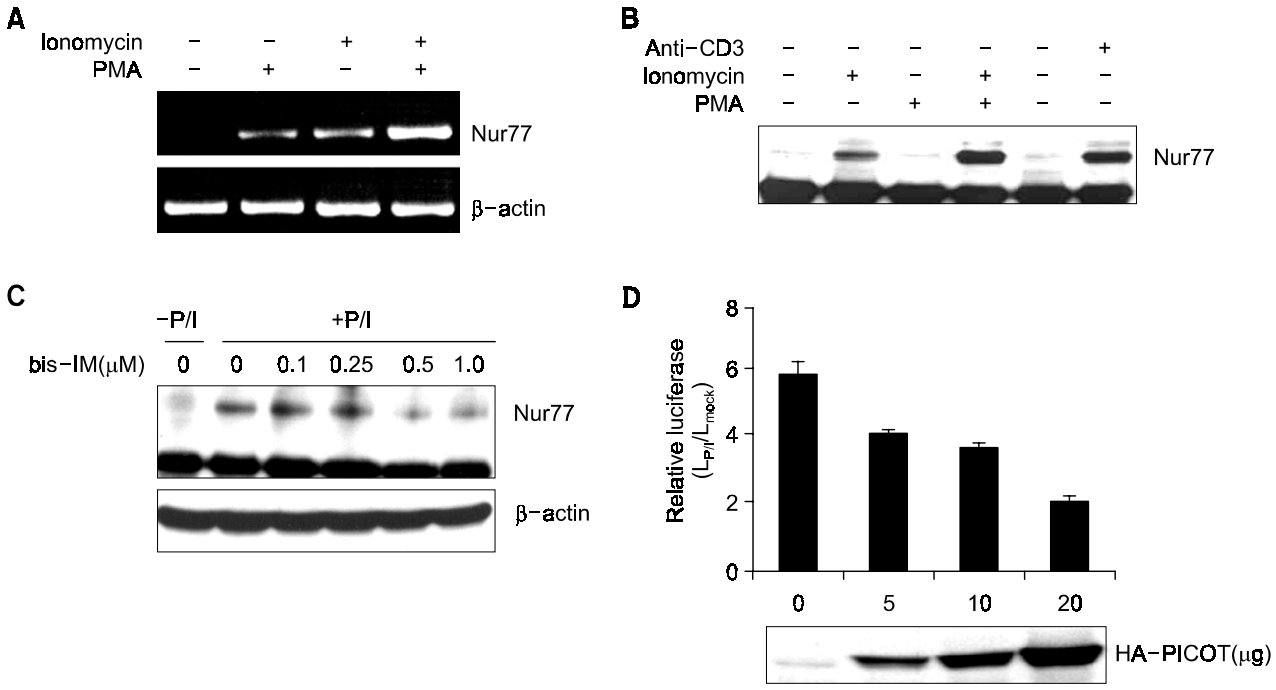

Figure 1. PKCθ is involved in Nur77 induction. PMA synergizes with ionomycin for the induction of Nur77 at the (A) mRNA and (B) protein level in DO11.10 T cells. DO11.10 T cells (10^7 cells/lane) were treated with ionomycin (1 μM), PMA (40 nM), or both or anti-CD3 monoclonal antibody for 3 h. RT-PCR was performed using extracted total RNAs. For immunoprecipitation, cell lysates were incubated with anti-Nur77 antibody for 2 h. Immunoprecipitates were subjected to 8% SDS-PAGE, transferred to nitrocellulose membrane, then western blotted with anti-Nur77 antibody. (C) A PKC inhibitor, Bisindolylmaleimide I (bis-IM) reduces the induction of Nur77. DO11.10 T cells were pretreated with varying concentrations of bis-IM for 30 min, followed by addition of PMA and ionomycin for another 3 h. The samples were processed as in (B). (D) PICOT, an endogenous PKCθ inhibitory protein also reduces the induction of Nur77. T cells were transfected with pNur77 (-337)-luc reporter gene, pEFPKCθ (A148/E), and increasing amounts of pSG5-HA-PICOT. PMA and ionomycin were added 16 h post-transfection. Luciferase activities were normalized with protein concentrations.

determine whether PKCθ is involved in Nur77 induction, we transiently transfected DO11.10 cells with HA-tagged PICOT, an endogenous PKCθ-specific inhibitor along with Nur77 promoter-driven luciferase reporter plasmid (Witte *et al.,* 2000). Similar to bis-IM, PICOT also inhibited the activation of the Nur77 reporter gene in a dose-dependent fashion, indicating that PKCθ serves as an activator for Nur77 induction in T cell (Figure 1D).

Mapping of PKCθ-responsive element in Nur77 promoter

Although PMA can induce mRNA of Nur77, its effect was transient (Figure 1A). Previously, the PMA response can be observed using a Nur77-luciferase reporter gene (Youn *et al.,* 1999). To further increase the sensitivity of the Nur77 reporter assay to PKC signaling, we overexpressed a constitutively active form of PKCθ that harbors a mutation of Ala148 to Glu. It was found that overexpression of PKCθ (A148/E) is sufficient to activate the Nur77-luciferase reporter to a level well above the background. Using this assay, we investigated the PKCθ-responsive DNA element in Nur77 promoter by transient transfection of DO11.10 cells with serial deletion mutants of Nur77 promoter-luciferase reporter plasmids along with the constitutively-active PKCθ (A148/E) (Figure 2A). The deletion of the Nur77 promoter to -337 had no effect on PKCθ-mediated reporter activities. Further deletion to -250 results in a less than 50% decrease in Nur77 promoter activity. There are two calcium-dependent MEF2 responsive elements between -297 and -288, and -262 and -253, indicating that MEF2D is, slightly, but not critically, regulated by PKCθ. Importantly, deletion of nucleotides -250 to -216 resulted in a dramatic decrease in Nur77-luciferase reporter gene activity, indicating that PKCθ-responsive element is localized between -250 and -226 bp. This region contains a putative AP-1 like element (TGCGTCA, -234 and -228) even though it is not a perfect match to AP-1 consensus sequence. To further confirm the role of the newly identified AP-1 like element in PKCθ-mediated Nur77 induction, we generated a mutant reporter plasmid of AP-1 like responsive element by converting TGCGTCA (wild type) to TGCGTTG (mutant) (Figure 2B). Mutation of AP-1 like site results in dramatic decrease of Nur77

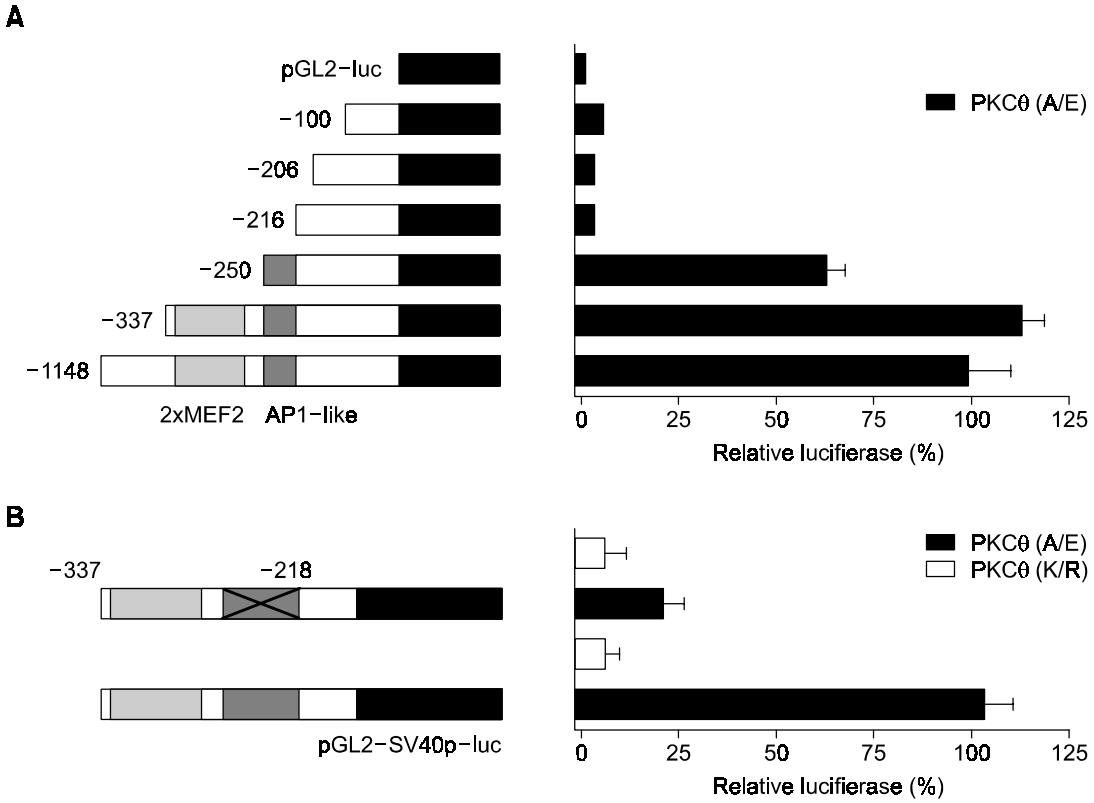

Figure 2. PKCθ activates the Nur77 promoter through the AP1-like element. (A) Mapping of PKCθ-responsive element on Nur77 promoter. (B) AP1-like element is important for PKCθ-mediated activation of Nur77. DO11.10 cells were transfected with constitutively-active PKCθ along with deletion mutants of Nur77 promoters [pNur77 (-337 to -216/WT)-luc or (pNur77 (-337 to -216/Mut)-luc).

promoter activity, indicating that AP-1 like element is crucial for PKCθ-mediated induction of Nur77.

When we performed gel shift assay with ^{32}P-labelled AP-1 like probe derived from the PKCθ-response element in the Nur77 promoter, we detected a single binding protein from nuclear extracts prepared from cells with or without prior treatment with PMA and ionomycin (Figure 3A). To identify the protein that is bound to the AP-1 like element, we performed supershift assay with either anti-c-jun or anti-JunD antibody. Treatment with anti-JunD antibodies leads to the appearance of a super-shifted band (Figure 3A), indicating that JunD is part of the complex that bound to the AP-1 like element. In contrast, anti-c-Jun antibody did not cause any super-shift of the band. It is worth noting that the protein-DNA complex did not change as a result of stimulation with PMA and ionomycin, consistent with the fact that JunD is constitutively expressed in T cells. Independently, we also determined the binding of JunD to the AP-1 like element using AP-1 like biotinylated oligonucleotide (Figure 3B).

PKCθ indirectly phosphorylates JunD through the activation of MAP kinases

As PKCθ is a protein kinase, we tested if PKCθ can phosphorylate JunD by transient transfection of HA-JunD along with PKCθ (A148/E). Cell lysates were immunoprecipitated with anti-HA antibody and then probed with either anti-HA or anti-phospho-JunD S100) antibody. Coexpression of the constitutively active form of PKCθ caused a significant level of phosphorylation of JunD (Figure 4A). The lack of a consensus phosphorylation site in JunD for PKCθ prompted us to test other kinases as well as PKCθ *in vitro*. We incubated recombinant GST-JunD with immunoprecipitates of anti-PKCθ, anti-JNK, or anti-ERK antibody, respectively, from PKCθ (A148/E)- activated cell lysates. PKCθ can not directly phosphorylate JunD, but ERK and JNK can both directly phosphorylate JunD (Figure 4B).

Menin represses PKCθ/p300-mediated activation of JunD in the Nur77 promoter

That JunD, unlike c-Jun, is not inducible in T cells and its binding to the PKCθ-responsive element in

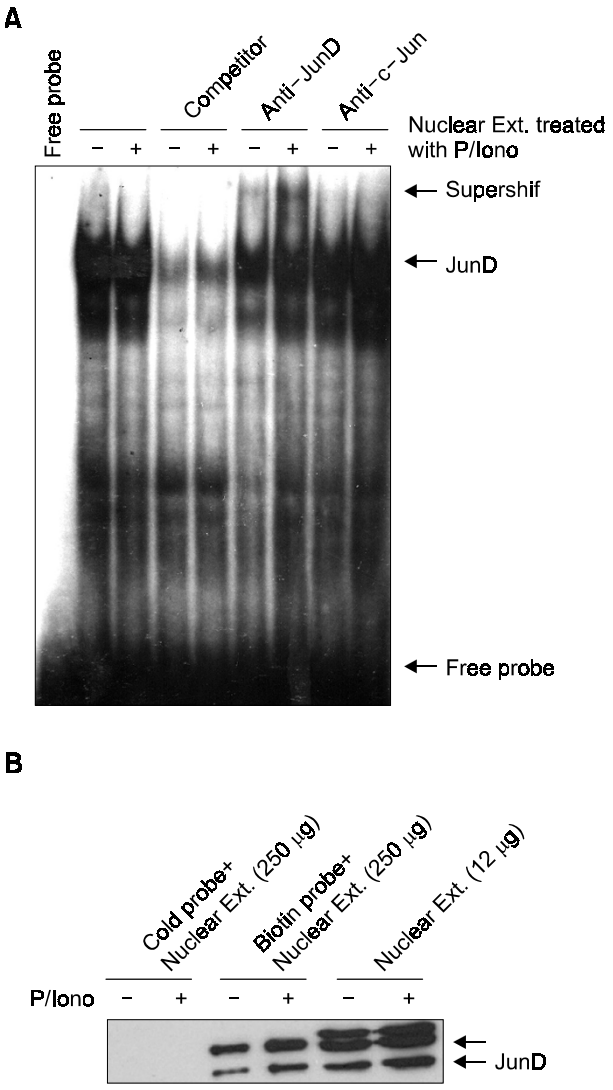

Figure 3. JunD binds to the AP1-like element in Nur77 promoter. (A) Electrophoretic mobility shift assay. Nuclear extracts (5 μg) prepared from cells treated with PMA and ionomycin, or none, were incubated with ^{32}P-labelled probe. Samples were subjected to 4% polyacrylamide gel electrophoresis and the binding of JunD was detected by autoradiography. For supershift assay, anti-JunD or anti-c-jun antibody was preincubated with the nuclear extracts prior to addition of ^{32}P-labelled probe. (B) Biotin-streptavidin pull-down assay. Nuclear extracts (250 μg) from naïve or PMA/ionomycin-treated DO11.10 cells were incubated with biotin-labelled probe for 2 h in the presence of stretavidin-beads, washed with washing buffer. Boiled samples were loaded into 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-JunD antibody.

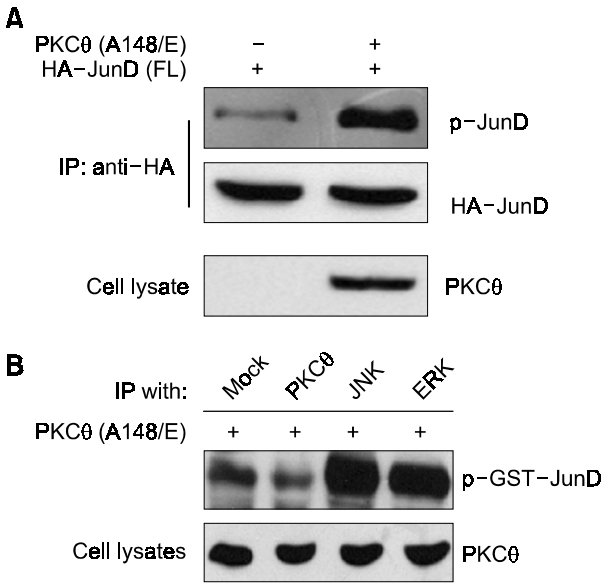

Figure 4. PKCθ regulates JunD. (A) PKCθ causes phosphorylation of JunD at serine 100. Cells were transfected with pSG-HA-JunD alone or along with pEF-PKCθ (A148/E). Immunoprecipitates of HA-JunD was immunoblotted with anti-phospho-JunD (S100) antibody. (B) Phosphorylation of JunD by PKCθ-activated MAP kinases. Cells were transfected with pEF-PKCθ (A148/E) followed by immunoprecipitations with anti-PKCθ, anti-JNK, and anti-Erk antibadies. GST-JunD was incubated with each of immunoprecipitates. Phosphorylation of GST-JunD was determined by immunoblotting with anti-phospho-JunD (S100) antibody.

the Nur77 promoter is not affected by PKC signal is reminiscent of MEF2. As MEF2 undergoes a signal-dependent switch from a transcriptional repressor to an activator by dissociation from corepressor complexes and association with coactivators, we speculated that JunD might also regulate Nur77 promoter in a similar manner. In fact, Menin, identified as a target gene of multiple endocrine neoplasia type I, has been known to bind to JunD and repress its transcriptional activity (Aggarwal *et al.,* 1999). The Menin-mediated repression of JunD can be reversed by trichostatinA (Gobl *et al.,* 1999), suggesting that Menin may be exerting its transcriptional repression effect on JunD by recruiting histone deacetylases. Recently we showed that Menin serves as a JunD repressor through the recruitment of an mSin3A-histone deacetylase complex (Kim *et al.,* 2003). However, it remains unclear how Menin in T cell is still associated with HDACs to repress JunD-driven Nur77 expression. To address this question, we transiently transfected Jurkat cells with Nur77 promoter-driven reporter gene along with the increasing amount of Menin (Figure 5A). PKCθ (A148/E) increases the Nur77 reporter activity, whereas increase of Menin gradually decreases the PKCθ (A148/E)-mediated Nur77 reporter activity, supporting that Menin represses PKCθ activation of Nur77 in T cells.

We investigated the interaction of JunD with p300 by transient transfection of HA-tagged JunD (pSG-HA-JunD) along with either N-terminal domain of p300 (pcdef3-flag-p300(1-682)) or C-terminal domain of p300 (pcdef3-flag-p300(1737-2414)). Under

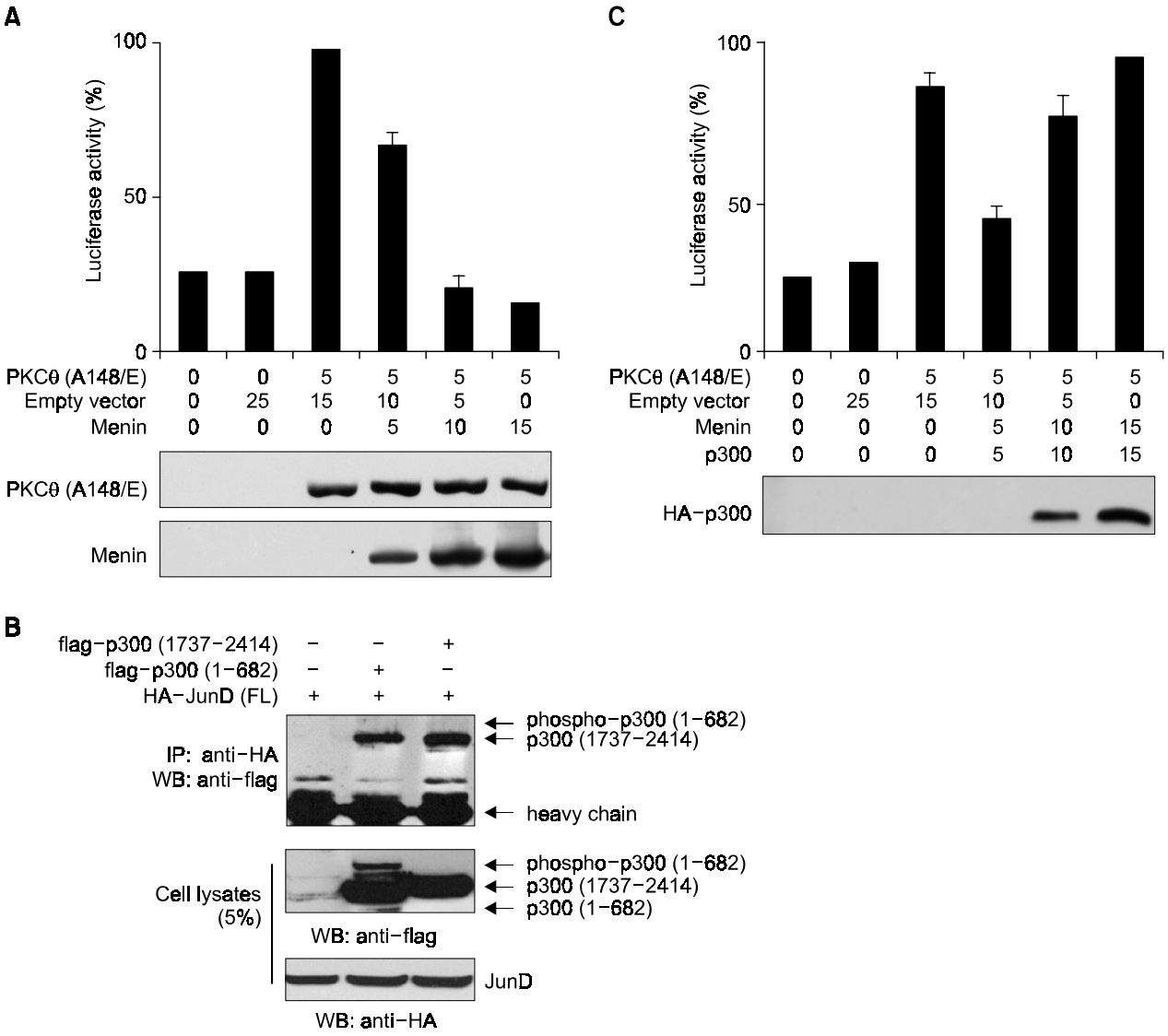

Figure 5. Menin represses PKCθ/p300-mediated activation of JunD in T cell. (A) Menin represses PKCθ-mediated transactivation of JunD under Nur77 promoter-driven luciferase reporter gene. (B) JunD binds to both of N- and C-terminal domains of p300. Cells were transfected with HA-JunD along with flag-tagged p300 (1-682) or flag-tagged p300 (1737-2414). Immunoprecipitates with anti-HA antibody were immunoblotted with anti-flag (M2) antibody. (C) p300 restores Menin-repressed JunD transcriptional activity. Jurkat cells were transfected with different combinations of expression vectors as described in panel. Reporter gene activities were measured and normalized with protein concentration in (A) and (C). This represents the average of three different experiments.

serum-enriched conditions, HA-JunD interacts with both of N-terminal and C-terminal of p300 (Figure 5B). Next we examined whether overexpression of p300 overcomes Menin-repressing activity of JunD using reporter gene assay. Transfection of PKCθ alone significantly increases Nur77 reporter gene activity, while cotransfection of Menin decreases reporter gene activity. However, increasing amount of p300 restores Menin-repressed JunD transcriptional activity (Figure 5C), implicating that p300 competes against Menin-HDAC complexes for binding to JunD.

Discussion

Nur77 family of transcription factor has been known to play a pivotal role in TCR-mediated apoptosis in immature T cells. Usually TCR signaling pathway bifurcates into two major signaling pathways in the cytosol, calcium and PKC pathways. Of two signals, calcium signal is sufficient to induce low level of Nur77 expression (Figure 1A). MEF2D has been shown to mediate calcium-dependent induction of Nur77 (Woronicz *et al.,* 1995). MEF2D is constitutively bound to the Nur77 promoter, switching bet-

ween an "off" state in the absence of an appropriate TCR signal and an "on" state in the presence of TCR signaling. We recently found that Cabin1 represses MEF2D by recruiting mSin3-histone deacetylase (Youn *et al.,* 1999; Youn and Liu, 2000). Repression of MEF2D by Cabin1 is sensitive to calcium signaling. Calcium-activated calmodulin (CaM) binds to MEF2D and release Cabin1-HDAC complexes from MEF2. In addition to Cabin1, MITR and Class II HDACs (HDAC4/5/7/9) also repress MEF2D transcriptional activity (Miska *et al.,* 1999; Sparrow *et al.,* 1999; Wang *et al.,* 1999; Dressel *et al.,* 2001). Class II HDACs bind to MEF2 through their N-terminal domain and directly inhibit MEF2 transcriptional activity through their C-terminal of intrinsic HDAC catalytic domain. MITR, lacking in HDAC catalytic domain, has been shown to repress MEF2 by recruitment of CtBP through PXDLR motif (Zhang *et al.,* 2001). We provided evidence that all MEF2 repressors are sensitive to calcium signaling by showing that MEF2-binding domain of these repressors share a CaM-binding domain and CaM releases these repressors from MEF2 similar to Cabin1 (Youn and Liu, 2000; Youn *et al.,* 2000b).

While much is known about calcium signaling during Nur77 expression in T cells, how PKC pathway activates Nur77 transcription remained largely unknown. It has been shown that PKCθ, a predominant isoform of PKC in T cells, activates AP-1 and NF-κB transcription factors in IL-2 production (Baier-Bitterlich *et al.,* 1996; Sun *et al.,* 2000). However, there are neither NF-κB response element nor exact AP-1 element, instead, four AP-1-like elements (TGCGTCA; NAP1, -68 to -62; NAP2, -39 to -33; NAP3, -212 to -206, NAP4, -232 to -226) in the murine Nur77 promoter. In pheochromocytoma-derived cell line PC12, NGF and membrane depolarization activate Nur77 through binding of JunD to two AP-1-like elements (NAP1 and NAP2), both of which are closest to TATA box (Yoon and Lau, 1994). Moreover, the Tax viral transactivator induces Nur77 by activation of JunD through the same NAP1 and NAP2 (Liu *et al.,* 1999). However, PMA-activation of Nur77 in T cells requires a quite different promoter sequence between -378 to -162, even though the exact PKC-responsive element in this region has not been confirmed yet (Liu *et al.,* 1994; Woronicz *et al.,* 1994). We identified the PKCθ-responsive element for Nur77 induction in T cells as the AP-1-like element (NAP4, -232 to -226), which is in close proximity to calcium-dependent MEF2-responsive element (Figure 2). We confirmed that JunD specifically binds to NAP4 and is activated indirectly by PKC through phosphorylation of JunD by MAP kinases (JNK and ERK) (Figure 5). In ovarian cells, calcium-dependent activation of ERK mediates JunD phosphorylation and Nur77 induction by prostaglandin (Stocco *et al.,* 2002). In this case, JunD induces the Nur77 through NAP1 and NAP2 elements. Interestingly, we found that PKCθ specifically synergizes with calcium to induce Nur77 expression only through NAP4, but not through other NAPs in Nur77 promoter, probably due to that JunD may cooperate with MEF2D since NAP4 is localized near the MEF2-responsive element. It is known that p300 bridges two discrete transcription factors and augments their transcriptional activities by cooperative recruitment of two transcription factors through N-terminal and C-terminal domain of p300 (Nakashima *et al.,* 1999). In fact, MEF2D binds to both of N-terminal and C-terminal domain of p300 (Youn *et al.,* 1999), and JunD also interacts with both of N-terminal and C-terminal domain of p300 (Figure 5B). Therefore, it is possible that p300 simultaneously interacts with both of MEF2D and JunD and integrates two transcriptional activities for Nur77 induction. It is very interesting that Nur77 induction in T cell differs from that in other cell types in that T cell requires MEF2 transcription factor for Nur77 expression, while other cell types just require JunD activity through NAP1 and NAP2 closest to TATA box (Youn and Lau, 1994; Stocco *et al.,* 2002).

JunD differs from other AP-1 family of transcription factors in that JunD is expressed constitutively at high levels and has no transforming activity (Hirai *et al.,* 1989; Ryder *et al.,* 1989; Hartl *et al.,* 1991). More distinctly, Menin, originally identified as a tumor suppressor, is a nuclear protein and specifically binds to JunD, but not other AP-1 family of transcriptional factors (c-Jun, JunB) and represses its transcriptional activity (Aggarwal *et al.,* 1999). The mechanism by which Menin represses JunD is still unclear. Recently, it was suggested that Menin inhibits JunD transcriptional activity by uncoupling of MAP kinase activation from MAP kinase-mediated phosphorylation of JunD (Gallo *et al.,* 2002). Recently, we provided direct evidence that Menin recruits HDACs through association with mSin3A, a general transcriptional corepressor (Kim *et al.,* 2003). On the contrary, PKC activation triggers phosphorylation of N-terminal domain of JunD. It is well known that N-terminal phosphorylation of c-Jun (S63 and S73) is required for CBP binding (Bannister *et al.,* 1995). Like c-jun, JunD has two conserved serine residues at its N-terminus. Overexpression of constitutively-active PKCθ triggers phosphorylation of N-terminal serine (100) residue, which probably mediates recruitment of p300 and enhances JunD transcriptional activity. Based on the reporter gene assay, increasing amount of p300 restores Menin-repressed JunD activity. However, we still cannot explain the molecular mechanism by which JunD switches partners

from Menin-mSin3-HDAC to p300 (or CBP) upon TCR activation. As Menin-binding domain in JunD is different from the p300-binding domain, it is not likely that Menin directly competes p300 for JunD binding. In biochemical binding assay *in vitro*, we failed to detect competition between Menin and p300 for association with JunD (data not shown). How JunD changes chromatin modifiers upon TCR activation remains to be addressed in the future.

In conclusion, we have identified the major DNA response element to PKC signal as an AP-1 like element in the Nur77 promoter. We found that JunD is the predominant isoform of AP-1 family that is bound to this element. Similar to the calcium-sensitive transcription factor, JunD is capable of silencing the Nur77 promoter by recruiting the Menin-mSin3-HDAC complex or activating the promoter by recruiting p300. The simultaneous existence of two HDAC containing complexes on the Nur77 promoter though MEF2 and JunD, respectively, may serve to ensure complete silencing of the Nur77 promoter in the absence of an appropriate TCR signal.

Acknowledgement

We are grateful to Drs. A. Altman, S. K. Agarwal, C. X. Zhang and A. Gobl for DNA constructs. This work was supported by grants from Cancer Research Institute, Seoul National University College of Medicine (CRI-04-6), Seoul National University Hospital (03-2004-010-0), Korean Science & Engineering Foundation (KOSEF, R01-2002-000-00242-0, H.-D. Y).

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