Protein Kinase D1 Phosphorylates HDAC7 and Induces Its Nuclear Export after T-cell Receptor Activation*

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Histone acetylation and deacetylation are important in modifying chromatin structure and in regulating gene expression in eukaryotes. Mammalian histone deacetylases (HDACs)1 are divided into three classes based on their homology to yeast proteins. Class I HDACs are homologous to Rpd3; class II HDACs are related to Hda1; and class III HDACs are homologous to Sir2. Class II HDACs are further subdivided into class IIA (HDAC4, HDAC5, HDAC7, and HDAC9) and class IIB (HDAC6 and HDAC10) (reviewed in Ref. 1). The class IIA HDACs possess a conserved C-terminal catalytic HDAC domain and interact with myocyte enhancer factor 2 (MEF2) transcription factors through an N-terminal 17-amino acid motif. This interaction leads to the recruitment of class IIA HDACs to select promoters, where MEF2 is bound, resulting in the repression of its transcriptional activity. The repressive activity of class IIA HDACs is tightly regulated by nucleocytoplasmic shuttling and by their phosphorylation-dependent association with the intracellular 14-3-3 proteins (2–5). Phosphorylation of conserved residues in the N-terminal regions of HDAC4, HDAC5, HDAC7, and HDAC9 in response to cellular signals leads to interaction with 14-3-3 proteins, dissociation of the class IIA HDAC-MEF2 complexes, and a conformational change culminating in export from the nucleus (6, 7).

Given the central role of phosphorylation in the regulation of class IIA HDAC activity, there is considerable interest in the identification of the responsible kinase(s). It has been reported that the calcium/calmodulin-dependent kinase (CaMK) phosphorylates HDAC4, HDAC5, and HDAC9, leading to their export from the nucleus and to gene activation in skeletal and cardiac myocytes (6–11). However, the endogenous HDAC kinase activity in cardiac myocytes is resistant to pharmacological inhibitors of CaMK, suggesting that other kinases may be responsible for HDAC phosphorylation (12).

We recently reported that HDAC7 is expressed at high levels in thymocytes during the CD4+ CD8+ double-positive stage (14). In resting thymocytes, HDAC7 is localized in the cell nucleus and represses the expression of the orphan steroid nuclear receptor Nur77 by interacting with the transcription factor MEF2D, which binds constitutively to the nur77 promoter (13, 14). nur77, an immediate-early gene, is up-regulated in response to T-cell receptor (TCR) activation and has been implicated in the negative selection (apoptosis) of T cells (15, 16). After TCR activation, HDAC7 is exported to the cytoplasm, leading to the derepression of the nur77 promoter and the induction of apoptosis. Interestingly, mutation of three serine residues at the N terminus of HDAC7 inhibits its nucleocytoplasmic shuttling in response to TCR activation and also suppresses TCR-induced apoptosis (14). These observations indicate that an intracellular signaling pathway, originating at the TCR, is involved in the phosphorylation and subcellular localization of HDAC7 in thymocytes and that this signal controls the apoptosis of thymocytes in response to TCR activation.

T cells use a complex array of signal transduction pathways to control their proliferation, differentiation, survival, and apoptosis. TCR activation leads to the activation of phospholipase Cγ1, which in turn initiates the activation of two well characterized major signaling pathways. Phospholipase Cγ1 induces the cleavage of phosphatidylinositol in the plasma membrane, producing inositol triphosphate and diacylglycerol. Inositol polyphosphates increase intracellular calcium levels by binding to specific receptors in the endoplasmic reticulum, resulting in the activation of the calcineurin/NF-AT cascade. Diacylglycerol (DAG) induces a calcium-independent signal
transduction pathway, which comprises the activation of protein kinase C (PKC) and protein kinase D (PKD) signaling modules (reviewed in Ref. 17). PKD1, also called PKCγ, is a serine/threonine kinase that belongs to a new family of protein kinases with two other members, PKD2 and PKD3/PKCγ. PKD1, the main isoform expressed in T cells (18), is mainly activated by a phospholipase C-DAG-PKC signal transduction pathway (reviewed in Ref. 19). PKD1 activation is mediated by the PKC-dependent phosphorylation of Ser744 and Ser748 in the activation loop of its catalytic domain (20, 21). PKD1 is activated in B- and T-cells after engagement of their respective receptors (18, 22–24). However, the specific substrates for PKD and its exact function in lymphocytes remain unclear.

Here, we show that a calcium-independent signaling pathway is responsible for the nucleocytoplasmic shuttling of HDAC7 in a thymocyte hybridoma cell line (DO11.10) after TCR activation. Moreover, PKD1, which is activated after TCR engagement, interacts with and phosphorylates HDAC7, leading to its nuclear export and to the activation of Nur77 transcription. Finally, we show that PKD1 is activated by TCR activation in vivo in a mouse model of negative selection.

EXPERIMENTAL PROCEDURES

Plasmids—The pcDNA3.1-based expression vector for FLAG-tagged human HDAC7 has been described (25). C-terminal green fluorescent protein (GFP) fusions were constructed in pEGFP-N1 (Clontech). Deletion constructs of HDAC7 were generated by PCR and the cloning procedures described previously (25). Site-directed mutagenesis was performed with a QuikChange kit (Stratagene, La Jolla, CA). All mutations were verified by DNA sequencing. The glutathione S-transferase (GST) fusion proteins containing the N or C terminus of HDAC7 have been described (25). The luciferase reporter plasmid driven by the nur77 promoter (pNur77-Luc) was generated by cloning the -3800 to +87 genomic sequences of the nur77 promoter (a kind gift from Astar Winoto, University of California, Berkeley, CA) (13) into pG2L-Basic (Promega). Minimal wild-type and mutant ME2 reporter constructs (pME2WT-Luc and pME2mut-Luc) were as described (13). PKD1 expression vectors were provided by Dr. Alex Toker (Beth Israel Deaconess Medical Center, Boston, MA).

Cell Culture, Transfections, and Reporter Assay—DO11.10 T-cell hybridomas and 293T cells were grown at 37 °C in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 units/ml streptomycin/penicillin. DO11.10 cells were transfected with the Dual-Luciferase reporter assay system (Promega) using an elongation factor 1α promoter-driven Renilla luciferase expression vector as an internal control. 293T cells were transfected by the standard calcium phosphate precipitation method. For anti-CD3/CD28 antibody stimulation, monoclonal antibodies 500A2 and 37.51 were bound to the culture flask by incubating a 1:2000 dilution in phosphate-buffered saline overnight at 4 °C, followed by three rinses with phosphate-buffered saline. The antibody specific for PKD1 phosphorylated at Ser744 and Ser748 was from Sigma. Anti-mouse Nur77 antibody was from Pharmingen (San Diego, CA). The antibody specific for PKD1 phosphorylated at Ser744 and Ser748 was from Cell Signaling (Beverly, MA).

GST Fusion Proteins and Pull-down Assays—These assays were performed as described (28).

Peptide Injection of Mice—DO11.10 transgenic mice have been described previously (29). Balb/c wild-type mice and DO11.10 transgenic mice were injected intraperitoneally with 250 μl of a sterile 100 μM solution of ovalbumin peptide (ISQAVHAAHAEINEAGR). Mice were sacrificed at the indicated times after injection.

RESULTS

A Calcium-independent Pathway Regulates Nucleocytoplasmic Shuttling of HDAC7—Our previous work (14) has documented that signals emanating from the TCR lead to the phosphorylation of HDAC7 and to its export from the nucleus. Accordingly, treatment of the thymocyte hybridoma cell line DO11.10 with phorbol esters and calcium ionophores (PMA/ ionomycin), a treatment that mimics the two main pathways activated by the TCR, led to the nuclear export of an HDAC7-GFP fusion protein. In contrast, an HDAC7 mutant in which phosphorylation was prevented by substituting Ser155, Ser318, and Ser380 with alanines (HDAC7S155A,S318A,S380A) remained in the nucleus (Fig. 1).

Surprisingly, treatment with PMA alone (but not ionomycin) led to the nuclear export of HDAC7 (Fig. 1), suggesting that a calcium-independent pathway is responsible for the nucleocytoplasmic shuttling of HDAC7 after TCR activation. The HDAC7 mutant was also unresponsive to PMA treatment, indicating that the conserved serines in HDAC7 are potential phosphorylation targets for this intracellular signaling cascade.

A PKC/PKD-dependent Pathway Regulates Nucleocytoplasmic Shuttling of HDAC7 after TCR Activation—To further define the factors involved in the nucleocytoplasmic shuttling of HDAC7, we used a number of specific inhibitors of distinct signaling pathways. Before PMA was added, DO11.10 cells expressing HDAC7-GFP were treated for 30 min with G66976, an inhibitor that targets both calcium-dependent PKC isoforms and PKD; with G66983 and GF109203X, two general inhibitors of PKC’s; with KN62, a specific inhibitor of CaMK; or with cyclosporin A (CsA), a specific inhibitor of calcineurin. Pre-treatment with G66976, G66983, and GF109203X completely inhibited HDAC7 nuclear export mediated by PMA (Fig. 2). In contrast, KN62 and CsA, which inhibit calcium-dependent pathways, had no effect on the cellular distribution of HDAC7 in response to PMA. Importantly, similar results were obtained after cross-linking the T-cell antigen receptor with anti-CD3/CD28 antibodies, a more physiologically relevant stimulus (Fig. 2). These observations indicate that CaMK and calcineurin are not involved in the nucleocytoplasmic shuttling of
HDAC7 after TCR activation and also that a PKC/PKD-dependent mechanism is responsible for the nucleocytoplasmic shuttling of HDAC7 after TCR activation.

A PKC/PKD-dependent Pathway Controls Nur77 Expression and Transcriptional Activation after TCR Engagement—In view of the key role of HDAC7 in regulating Nur77 expression after TCR activation, we tested the role of a PKC/PKD signaling pathway in Nur77 induction after TCR activation. In agreement with the results shown in Fig. 2, pretreatment with the inhibitors G6976, G6983, and GF109203X suppressed the induction of Nur77 protein expression in response to PMA, whereas KN62 and CsA surprisingly potentiated the effect of PMA (Fig. 3A). Similar results were obtained in cells transfected with a construct containing the nur77 promoter driving a luciferase reporter. The PMA-mediated transcriptional activation of the nur77 promoter was inhibited by the PKC inhibitors and potentiated by KN62 and CsA (Fig. 3B).

The effects of the same inhibitors were tested after the induction of Nur77 expression by anti-CD3/CD28 antibodies. Pretreatment of DO11.10 cells with the PKC/PKD inhibitor (G6976) abolished the induction of Nur77 at the level of both protein expression and promoter induction (Fig. 3, C and D). The other inhibitors (G6983, GF109203X, and CsA) partially suppressed Nur77 expression and its promoter (Fig. 3, C and D). The calmodulin inhibitor (KN62) had no effect on the induction of Nur77 after TCR activation, further confirming that CaMKs are not involved in the HDAC7-dependent regulation of Nur77 by PMA or the TCR (Fig. 3, C and D).

PKD1 Is Activated in Thymocyte Hybridoma Cells after TCR Engagement—Next, we analyzed the amino acid sequences surrounding the conserved serines in HDAC7 for homology to known consensus protein kinase sites. The three conserved serines in HDAC7 (Ser155, Ser318, and Ser448) and other class IIa HDACs are consensus sites for CaMK ((R/K)XX(S/T)). However, as shown above, chemical blockage of CaMK did not suppress HDAC7 nuclear export after TCR activation. Interestingly, leucine was present at position −5 relative to each serine (Fig. 4A), a requirement for substrates of PKD1 (30).

To determine whether PKD1 is activated by antigen receptor signals, we used an antibody specific for the active/phosphorylated...
lated form of PKD1 (Ser744 and Ser748). Treatment of DO11.10 cells with PMA/ionomycin induced PKD1 phosphorylation in a time-dependent manner (Fig. 4B). PMA alone (but not ionomycin) had a similar effect (Fig. 4B), demonstrating that a calcium-independent mechanism is involved. Cell treatment with anti-CD3/CD28 antibody also activated PKD1 (Fig. 4B). To further confirm the activation of PKD1 in DO11.10 cells, we performed an in vitro kinase assay. The cells were treated with PMA/ionomycin, and endogenous PKD1 was immunoprecipitated with anti-PKD1 antibody, followed by a kinase assay using myelin basic protein as substrate. Endogenous PKD1 was activated after TCR activation, as indicated by the PKD1 autophosphorylation band and the phosphorylation of myelin basic protein (Fig. 4C). These results demonstrate that PKD1 is

FIG. 3. A PKC-dependent pathway induces Nur77 expression after TCR activation. A, total cell lysates were prepared from DO11.10 cells pretreated with 3.5 μM Go6976, 3.5 μM Go6983, 3 μM GF109203X, 1 μM CsA, or 5 μM KN62 for 30 min and then left untreated or treated with PMA for 2 h. Cell lysates were analyzed by Western blotting with antisera against Nur77 and tubulin. B, DO11.10 cells were transfected with the pNur77-Luc reporter construct. Thirty-six hours after transfection, cells were pretreated with 3.5 μM Go6976, 3.5 μM Go6983, 3 μM GF109203X, 1 μM CsA, or 5 μM KN62 for 30 min, followed by stimulation for 4 h with PMA. Luciferase activities are expressed relative to the activity of pNur77-Luc in untreated DO11.10 cells, which was given an arbitrary value of 1. Values are the mean of three independent experiments, each performed in triplicate. RLU, relative luciferase units. C, total cell lysates were prepared from DO11.10 cells pretreated as described for A and then left untreated or treated with anti-CD3/CD28 antibodies for 2 h. Cell lysates were analyzed by Western blotting with antisera against Nur77 and tubulin. D, DO11.10 cells were transfected with the pNur77-Luc reporter construct. Thirty-six hours after transfection, cells were pretreated as described for A and then stimulated for 4 h with anti-CD3/CD28 antibodies. The activity of the nur77 promoter is expressed as in B. Values are the mean of three independent experiments, each performed in triplicate.

FIG. 4. PKD1 is activated in DO11.10 cells after TCR engagement. A, the amino acid sequence surrounding the conserved serines in class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are aligned. B, DO11.10 cells were treated with PMA/ionomycin, PMA, ionomycin, or anti-CD3/CD28 antibodies. Total cell lysates were prepared 0, 10, 30, and 60 min after activation and analyzed by Western blotting with an antibody specific for PKD1 phosphorylated at Ser744 and Ser748 (Phospho-PKD1) and an antibody specific for total PKD1. Tubulin levels were measured by Western blotting of the same samples. C, DO11.10 cells were treated with PMA/ionomycin, and total cell lysates were prepared 0, 10, 30, and 60 min after activation. PKD1 activity was determined by immunoprecipitation of endogenous PKD1 from the cell lysates, followed by a kinase assay using the substrate myelin basic protein (MBP).
activated in DO11.10 cells by TCR activation independently of calcium signaling.

**PKD1 Interacts with and Phosphorylates HDAC7**—To test whether HDAC7 interacts with PKD1, we incubated GST fusion proteins containing the N terminus (GST-HDAC7(N-ter)) or the C terminus (GST-HDAC7(C-ter)) of HDAC7 after expression in bacteria with extracts of DO11.10 cells, either untreated or treated with PMA/ionomycin. Immunoblotting with an antibody specific for PKD1 revealed that endogenous PKD1 bound to GST-HDAC7(N-ter), but not to GST-HDAC7(C-ter) (Fig. 5A). HDAC7 bound to PKD1 present in resting or activated extracts (PMA/ionomycin) (Fig. 5A), suggesting that enzymatic activation of PKD1 is not necessary for HDAC7 binding. To further confirm the interaction of HDAC7 with PKD1, FLAG-HDAC7 and HA-PKD1 expression plasmids were coexpressed in 293T cells. FLAG-HDAC7 was immunoprecipitated, and the immunoprecipitate was probed for the presence of PKD1. Both proteins interacted, regardless of the order of immunoprecipitation (Fig. 5B).

To investigate whether PKD1 phosphorylates HDAC7 directly, we immunoprecipitated endogenous PKD1 from DO11.10 cells, untreated or treated with PMA/ionomycin. Immunoblotting with an antibody specific for PKD1 revealed that endogenous PKD1 bound to GST-HDAC7(N-ter), but not to GST-HDAC7(C-ter) (Fig. 5A). HDAC7 bound to PKD1 present in resting or activated extracts (PMA/ionomycin) (Fig. 5A), suggesting that enzymatic activation of PKD1 is not necessary for HDAC7 binding. To further confirm the interaction of HDAC7 with PKD1, FLAG-HDAC7 and HA-PKD1 expression plasmids were coexpressed in 293T cells. FLAG-HDAC7 was immunoprecipitated, and the immunoprecipitate was probed for the presence of PKD1. Both proteins interacted, regardless of the order of immunoprecipitation (Fig. 5B).

**PKD1 Controls HDAC7 Nucleocytoplasmic Shuttling**—Because HDAC7 Ser155, Ser318, and Ser448 were required for nuclear export after TCR activation, we tested whether PKD1 could regulate the subcellular distribution of HDAC7. DO11.10 cells were cotransfected with the HDAC7-GFP or HDAC7ΔP-GFP expression vector and, where indicated, with a plasmid expressing the wild-type (PKD1 wt), constitutively active (PKD1 SS/EE), or kinase-inactive (PKD1 SS/AA) form of PKD1. Thirty-six hours after transfection, cells were left untreated or treated with PMA, and the intracellular location of GFP fusion proteins was monitored by confocal immunofluorescence microscopy after 1 h of treatment. One or two cells are shown for each experimental condition. They are representative of >90% of the total population.
PKD1 Controls HDAC7 Nucleocytoplasmic Shuttling

PKD1 overexpression can block the repressive activity of HDAC7 (75%) induced by PMA/ionomycin (Fig. 7B). Overexpression of constitutively active PKD1 reversed the repressive effect of wild-type HDAC7 (Fig. 7B). Importantly, PKD1 SS/EE had no effect on the transcriptional repression mediated by the HDAC7AP phosphorylation mutant (Fig. 7B). To confirm that MEF2 is a target of the PKD1-dependent activation, we cotransfected DO11.10 cells with a reporter construct containing four MEF2D-binding sites upstream of a minimal promoter (pMEF2wt-Luc) and the PKD1 SS/EE expression vector. A construct containing mutant MEF2D-binding sites served as a control (pMEF2mt-Luc). PMA/ionomycin increased the activity of the pMEF2wt-Luc reporter construct by 18-fold, but had no effect on the pMEF2mt-Luc construct (Fig. 7C). Overexpression of PKD1 SS/EE caused a 12-fold activation of pMEF2wt-Luc under basal conditions and further increased its activity in response to PMA/ionomycin (Fig. 7C). As predicted, active PKD1 had no effect on pMEF2mt-Luc (Fig. 7C).

PKD1 Is Activated during Negative Selection of T cells—Our observations indicated that PKD1 regulated gene expression in thymocytes during T-cell activation in an MEF2- and HDAC7-dependent manner. To test the relevance of these observations in vivo, we examined the status of PKD1 in a mouse model of negative selection. DO11.10 transgenic mice express a class II

PKD1 Overexpression Activates Nur77 Expression through MEF2—To determine whether PKD1-induced HDAC7 nuclear export is sufficient to activate the nur77 promoter, we cotransfected DO11.10 cells with a nur77 promoter-reporter construct and a vector encoding constitutively active PKD1 (PKD1 SS/EE). PKD1 SS/EE increased the basal transcriptional activity of the nur77 promoter by ~5-fold; activation was more modest in the presence of PMA/ionomycin (Fig. 7A). We reported previously that HDAC7 overexpression can block the TCR-mediated activation of the nur77 promoter (14). To test whether PKD1 overexpression can block the repressive activity of HDAC7, we transfected DO11.10 cells with the HDAC7 or HDAC7AP expression vector. As expected, overexpression of HDAC7 inhibited the transcriptional activation of Nur77 (75%) induced by PMA/ionomycin (Fig. 7B). Overexpression of constitutively active PKD1 reversed the repressive effect of wild-type HDAC7 (Fig. 7B). Importantly, PKD1 SS/EE had no effect on the transcriptional repression mediated by the HDAC7AP phosphorylation mutant (Fig. 7B). To confirm that MEF2 is a target of the PKD1-dependent activation, we cotransfected DO11.10 cells with a reporter construct containing four MEF2D-binding sites upstream of a minimal promoter (pMEF2wt-Luc) and the PKD1 SS/EE expression vector. A construct containing mutant MEF2D-binding sites served as a control (pMEF2mt-Luc). PMA/ionomycin increased the activity of the pMEF2wt-Luc reporter construct by 18-fold, but had no effect on the pMEF2mt-Luc construct (Fig. 7C). Overexpression of PKD1 SS/EE caused a 12-fold activation of pMEF2wt-Luc under basal conditions and further increased its activity in response to PMA/ionomycin (Fig. 7C). As predicted, active PKD1 had no effect on pMEF2mt-Luc (Fig. 7C).

PKD1 Is Activated during Negative Selection of T cells—Our observations indicated that PKD1 regulated gene expression in thymocytes during T-cell activation in an MEF2- and HDAC7-dependent manner. To test the relevance of these observations in vivo, we examined the status of PKD1 in a mouse model of negative selection. DO11.10 transgenic mice express a class II

Fig. 7. PKD1 is involved in Nur77 induction after TCR activation. A, DO11.10 cells were transfected with the pNur77-Luc plasmid and the indicated PKD1 expression vectors and activated with PMA/ionomycin for 4 h. Luciferase activity was measured in treated and untreated cells. Luciferase activities are expressed relative to the activity of pNur77-Luc in untreated DO11.10 cells, which has been given an arbitrary value of 1. The results represent the average of three independent experiments, each performed in triplicate. B, Luciferase activity was measured in treated and untreated cells. Luciferase activities are expressed relative to the activity of pNur77-Luc in untreated DO11.10 cells, which has been given an arbitrary value of 1. Values are the mean of three independent experiments, each performed in triplicate. C, Luciferase activity was measured in treated and untreated cells. Luciferase activities are expressed relative to the activity of either the pMEF2wt-Luc or pMEF2mt-Luc reporter construct. DO11.10 cells, which has been given an arbitrary value of 1. Values are the mean of three independent experiments, each performed in triplicate.
PKD1 Controls HDAC7 Nucleocytoplasmic Shuttling

**Fig. 9.** The signaling pathway responsible for nucleocytoplasmic shuttling of HDAC7 after TCR activation. In resting T cells, HDAC7 represses the transcriptional activation of Nur77 by binding to the transcription factor MEF2D. After TCR activation, PKD1 is activated, presumably by a phospholipase C (PLC)–DAG–PKC signal transduction pathway. PKD1 phosphorylates HDAC7, leading to its interaction with 14-3-3 proteins and to its export from the nucleus, resulting in the activation of Nur77 and the induction of apoptosis.

Major histocompatibility complex-restricted TCR specific for a peptide sequence derived from ovalbumin. CD4+ T-cells carrying this TCR undergo massive apoptosis (negative selection) after injection of the ovalbumin peptide in mice (29).

To assess the activation status of PKD1 in vivo, we injected wild-type or DO11.10 transgenic mice with the ovalbumin peptide. Nur77 expression was rapidly and specifically induced in DO11.10 mice, but not in wild-type controls (Fig. 8A). PKD1 was activated in thymocytes from DO11.10 transgenic mice after ovalbumin peptide injection, but not in control mice (Fig. 8B). The activation of PKD1 followed kinetics similar to that of Nur77 expression. Moreover, endogenous PKD1 immunoprecipitated from in vivo activated DO11.10 transgenic thymocytes could phosphorylate recombinant GST-HDAC7. These results demonstrate that PKD1 is activated during negative selection and could play a significant role in the observed derepression of Nur77 expression during this process.

**Discussion**

This study shows that the export of HDAC7 from the nucleus is induced by a calcium-independent mechanism after TCR activation. We have identified the serine/threonine kinase PKD1 as a key player in the nuclear-cytoplasmic transfer of HDAC7. PKD1 is activated in DO11.10 cells in a calcium-independent manner after TCR activation or PMA treatment. PKD1 interacts with and phosphorylates HDAC7, leading to its nuclear export and to the transcriptional activation of Nur77 (Fig. 9). In agreement with our previous observation (14) that HDAC7 plays a critical role in the transcriptional repression of the orphan receptor Nur77, PKD1 plays a critical role in the transcriptional regulation of Nur77. Importantly, a kinase-inactive form of PKD1 suppresses the nucleocytoplasmic transfer of HDAC7 in response to TCR activation. In agreement with these observations, we observed that PKD1 is activated during an in vivo model of negative selection of T cells, a process driven in part by Nur77.

Strong TCR activation leads to the induction of Nur77 in T-cell hybridomas and thymocytes, leading to apoptosis (15, 16). In vivo, Nur77 plays a major role in negative selection, an apoptotic process responsible for the elimination of developing self-reactive thymocytes (reviewed in Ref. 31). Two MEF2-binding sites in the nur77 promoter are critical for its regulation of Nur77 expression (13, 32). The transcriptional corepressor Cabin-1 represses MEF2-dependent Nur77 transcriptional activity by recruiting mSin3, HDAC1, and HDAC2 and by competing with the coactivator p300 for MEF2 binding (32). However, a mutant transgenic mouse expressing Cabin-1 lacking the MEF2-binding domain shows normal thymocyte development and apoptosis (33). This observation suggests that other proteins participate in the repression of Nur77 during thymocyte development and that the role of Cabin-1 might be restricted to a subpopulation of lymphocytes. Our observations are consistent with the model that HDAC7 contributes to the repression of Nur77 in developing thymocytes, either in cooperation with or independently of Cabin-1.

We recently reported that HDAC7 is the predominant class IIa HDAC in thymocytes (14). HDAC7 interacts directly with MEF2 and inhibits the expression of Nur77 through MEF2D in resting T cells. The repressive activity of HDAC7 is regulated at the level of its subcellular distribution between the nucleus and the cytoplasm. While in the nucleus of resting thymocytes, HDAC7 is presumably bound to MEF2 at promoter sites and participates in the repression of their transcriptional activity. TCR activation leads to the nuclear export of HDAC7 and the derepression of promoters previously repressed by HDAC7. Importantly, the subcellular localization of HDAC7 mutated at three serine residues (Ser155, Ser318, and Ser448) is not modified in response to TCR activation. This mutant remains in the nucleus after TCR activation and inhibits TCR-mediated apoptosis. These three serine residues are highly conserved among all class IIa HDACs, undergo reversible phosphorylation, and control the nucleocytoplasmic shuttling of these factors. Phosphorylation of these residues unmasks a nuclear export signal and leads to cytoplasmic localization of class IIa HDACs. Phosphorylated class IIa HDACs are bound to 14-3-3 proteins and are present in the cytoplasm dissociated from the N-Cor-SMRT-HDAC3 corepressor complex. These observations therefore identify the phosphorylation of HDAC7 and the kinase(s) that mediate this event as key in the transcriptional activation of the nur77 promoter and other promoters controlled by HDAC7.

CaMK can phosphorylate HDAC4 and HDAC5, and the expression of constitutively active CaMK induces nucleocytoplasmic shuttling in myocytes (6–11). However, the endogenous HDAC kinase activity in cardiac myocytes is resistant to pharmacological inhibitors of CaMK, suggesting that other kinases are involved in the phosphorylation and regulation of the subcellular distribution of HDACs in these cells (12). While this manuscript was in preparation, Vega et al. (34) reported that PKC and PKD are involved in the nuclear export of HDAC5 in cardiac myocytes in response to hypertrophic agonists. In that experimental system, both ionomycin and PMA independently induce HDAC5 nucleocytoplasmic shuttling. In contrast, we have reported that PMA alone (and not ionomycin) induced HDAC7 nucleocytoplasmic shuttling. These observations indicate that PKD1 is a novel regulator of the nucleocytoplasmic shuttling of class IIa HDACs and reveal an interesting divergence in the regulatory pathways controlling the nucleocytoplasmic shuttling of different class IIa HDACs. Other differences in nucleocytoplasmic shuttling between class IIa HDACs and between cell types have been noted. In transfectod fibroblasts, exogenously overexpressed HDAC5 is exclusively nuclear, whereas HDAC4 is cytoplasmic in 50–80% of cells, suggesting that HDAC4 and HDAC5 are differentially phos-
Phosphorylated in vivo (10). HDAC7 is predominantly nuclear in CV-1 and HeLa cells (4, 35), whereas it is distributed in both the nucleus and the cytoplasm of T cells (14).

PKD1 is a member of a new family of DAG-stimulated serine/threonine protein kinases that consists of three members: PKD1, PKD2, and PKD3. Based on the sequence similarity of their kinase domains, PKD family members are classified as a subgroup of the CaMKs (reviewed in Ref. 19). PKD family members share a common mechanism of activation, but vary in their tissue distribution and subcellular localizations. PKD1 is most abundant in hematopoietic cells and is the predominant PKD isoform expressed in T cells. PKD1 is activated by the TCR and is localized either at the plasma membrane or in the cytosol of lymphocytes (18). PKD1 targeted to either the membrane or the cytosol induces differentiation in transgenic mice reminiscent of β-selection: down-regulation of CD25 and up-regulation of CD2 and CD5 (18). Strikingly, membrane (but not cytosolic) PKD1 can induce the expression of CD8 and CD4 in RAG recombinase-null mice, and cytosolic (but not membrane) PKD1 can induce the expression of CD8 and CD4 in the regulation of CD2 and CD5 (18). Strikingly, membrane (but not cytosolic) PKD1 can induce the expression of CD8 and CD4 in RAG recombinase-null mice, and cytosolic (but not membrane) PKD1 suppresses Vβ-to-DJβ rearrangements of the TCR β-chain locus in wild-type T cells, suggesting their involvement in the signal mediating TCR allelic exclusion. The fact that HDAC7 is regulated by PKD1 suggests the intriguing possibility that the nucleocytoplasmic shuttling of HDAC7 could affect not only late events in thymocyte differentiation, such as negative and positive selection, but also earlier events, such as β-selection. Our observations suggest the existence of a nuclear form of PKD1 in activated thymocytes. Although this has not been formally demonstrated, we were able to co-immunoprecipitate HDAC7 and PKD1, indicating that the two proteins interact. In addition, PKD1 translocates to the nucleus under defined physiological conditions, e.g. in response to G protein-coupled signaling (36).

TCR engagement leads to the activation of a wide array of signaling events that mediate the transcriptional activation or repression of many target genes. Key in this process is the activation of phospholipase Cγ, which induces the production of inositol trisphosphate and DAG, resulting in an increase in intracellular calcium levels and in the activation of a number of kinases, including PKC. Accordingly, many of the events induced by TCR activation can be recapitulated by treating target cells with phorbol esters (a DAG mimic) and ionomycin (a calcium ionophore). Considerable experimental evidence suggests an important role for calcium signaling in Nur77 induction after TCR activation (13, 32). This activation is mediated in part through the recruitment of NF-AT and in part through the dissociation of Cabin-1 from MEF2D. In DO11.10 cells, the addition of CaS, an inhibitor of the calcium-dependent phosphatase calcineurin, partially inhibited the late expression of Nur77 in response to PMA/ionomycin or anti-CD3 antibody and also prevented subsequent cell death. However, it is also clear that calcium-independent processes play a critical role in activating the nur77 promoter. For example, treatment of DO11.10 cells with PMA alone induced early Nur77 expression. Calcium-independent signals activate the nur77 promoter in part by recruiting the extracellular signal-regulated kinase ERK5 to the promoter (37). The induction of HDAC7 nucleocytoplasmic shuttling by PKD1 phosphorylation in response to TCR activation represents a novel calcium-independent signal participating in the induction of the nur77 promoter.

Future efforts will be directed toward dissecting the molecular events linking TCR cross-linking and the activation of PKD1. It has been reported that PKD1 is a downstream target of PKCθ in COS-7 cells and Jurkat T-lymphocytes (23). PKCθ activation has been linked to the induction of apoptosis in double-positive (CD4+ CD8+) thymocytes in response to TCR cross-linking (38). A phospholipase Cγ-PKCθ axis could be a critical link between the TCR and PKD1. The involvement of a PKC in the nucleocytoplasmic shuttling of HDAC7 could also explain why general PKC inhibitors, such as Gö6983 and GF109203X, suppressed the cytoplasmic translocation of HDAC7 in response to TCR cross-linking (Fig. 2).

Our observations that PKD1 regulates Nur77 induction by phosphorylating HDAC7 indicate that PKD1 could be important in the negative selection of T cells. Indeed, PKD1 was activated in thymocytes from DO11.10 transgenic mice after the induction of apoptosis with a peptide specific for their TCR. This activation correlated with the induction of Nur77 and further supports its potential role in the negative selection of T cells. Future experiments examining the biological consequences of knocking down either the Pkd1 or Hdac7 gene will test these predictions and further define the role of PKD1 and HDAC7 in thymocyte development.

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REFERENCES

1. Verdin, E., Dequiedt, F., and Kasler, H. G. (2003) Trends Genet. 19, 286–293
2. Dressel, U., Bailey, P. J., Jurewicz, D., Downes, E., Evans, R. M., and Mascet, G. E. (2001) J. Biol. Chem. 276, 17007–17013
3. Grozinger, C. M., and Schreiber, S. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7835–7840
4. Kao, H. Y., Verdel, A., Tsai, C. C., Simon, C., Jugulion, H., and Khochk, S. (2001) J. Biol. Chem. 276, 47496–47507
5. Zhou, X., Marks, P. A., Rifkind, R. A., and Richon, V. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10572–10577
6. McKinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000) Nature 408, 106–111
7. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) Mol. Cell. Biol. 21, 6312–6321
8. Passier, R., Zeng, H., Frey, N., Naya, F. J., Nicol, R. L., McKinsey, T. A., Overbeek, P., Richardson, J. A., Grant, S. R., and Olson, E. N. (2000) J. Clin. Investig. 105, 11713–11720
9. Lu, J., McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2000) Mol. Cell 6, 233–244
10. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14400–14405
11. Lu, J., McKinsey, T. A., Nicol, R. L., and Olson, E. N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4070–4075
12. Zhang, C. L., McKinsey, T. A., Zhang, S., Antos, C. L., Hill, J. A., and Olson, E. N. (2002) Cell 110, 479–488
13. Woronicz, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994) J. Biol. Chem. 269, 35826–35835
14. Dequiedt, F., Kasler, H., Fischle, W., Kiermer, V., Weinstein, M., Herndier, B. G., and Verdin, E. (2003) Immunity 18, 687–698
15. Liu, Z. G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M., and Osborne, B. A. (1984) Nature 317, 281–284
16. Woronicz, J. D., Waldron, R. T., and Rozengurt, E. (1998) J. Biol. Chem. 273, 27662–27667
17. Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2003) J. Biol. Chem. 278, 29296–29305
18. Matthews, S. A., Dayalu, R., Thompson, L. J., and Scharenberg, A. M. (2003) J. Biol. Chem. 278, 9086–9091
19. Yuan, J., Bae, D., Cantrell, D., Nel, A. E., and Rozengurt, E. (2002) Biochem. Biophys. Res. Commun. 291, 444–452
20. Sidorenko, S. P., Law, C. L., Klaus, S. J., Chandran, K. A., Takata, M., Kurotski, T., and Clark, E. A. (1996) Immunity 5, 353–363
21. Fischle, W., Dequiedt, F., Filion, M., Hendzel, M. J., Voelter, W., and Verdin, E. (2001) J. Biol. Chem. 276, 35826–35835
22. Fischle, W., Emiliani, S., Hendzel, M. J., Nagase, T., Nomura, T., Voelter, W., and Verdin, E. (1999) J. Biol. Chem. 274, 11713–11720
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., and Verdin, E. (2001) Mol. Cell. Biol. 21, 479–488
25. Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z., and Cantley, L. C. (1997) J. Biol. Chem. 272, 952–960
26. Sohn, S. J., Rajpal, A., and Winoto, A. (2003) Curr. Opin. Immunol. 15, 209–216
32. Youn, H. D., and Liu, J. O. (2000) *Immunity* 13, 85–94
33. Esau, C., Boes, M., Youn, H. D., Tatterson, L., Liu, J. O., and Chen, J. (2001) *J. Exp. Med.* 194, 1449–1459
34. Vega, R. B., Harrison, B. C., Meadows, E., Roberts, C. R., Papst, P. J., Olson, E. N., and McKinsey, T. A. (2004) *Mol. Cell. Biol.* 24, 8374–8385
35. Kao, H. Y., Downes, M., Ordentlich, P., and Evans, R. M. (2000) *Genes Dev.* 14, 55–66
36. Rey, O., Zhukova, E., Sinnett-Smith, J., and Rozengurt, E. (2003) *J. Cell. Physiol.* 196, 483–492
37. Kasler, H. G., Victoria, J., Duramad, O., and Winoto, A. (2006) *Mol. Cell. Biol.* 26, 8382–8389
38. Asada, A., Zhao, Y., Komano, H., Kawai, T., Mukai, M., Fujita, K., Tozawa, Y., Iseki, R., Tian, H., Sato, K., Motegi, Y., Suzuki, R., Yokoyama, M., and Iwata, M. (2000) *Immunology* 101, 309–315