PKD2 interacts with Lck and regulates NFAT activity in T cells

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Protein kinase D2 (PKD2) is a member of the PKD serine/threonine protein kinase family that has been implicated in the regulation of a variety of cellular processes including proliferation, survival, protein trafficking and immune response. In the present study, we report a novel interaction between PKD2 and Lck, a member of the Src tyrosine protein kinase family that is predominantly expressed in T cells. This interaction involved the C-terminal kinase domains of both PKD2 and Lck. Moreover, co-expression of Lck enhanced the tyrosine phosphorylation of PKD2 and increased its kinase activity. Finally, we report that PKD2 enhanced T cell receptor (TCR)-induced nuclear factor of T cell (NFAT) activity in Jurkat T cells. These results suggested that Lck regulated the activity of PKD2 by tyrosine phosphorylation, which in turn may have modulated the physiological functions of PKD2 during TCR-induced T cell activation. [BMB reports 2009; 42(1): 35-40]

INTRODUCTION

Induction of cellular growth and the functional repertoire of resting T cells requires engagement of the T cell receptor (TCR)/CD3. One of the earliest signaling events in T cell activation via the TCR/CD3 complex is the activation of the Src and Syk families of protein tyrosine kinases (PTKs), which leads to the phosphorylation of many cellular proteins (1). Various members of the Src family of protein tyrosine kinases have been implicated in the regulation of cell growth and differentiation. Lck is a member of the Src family of kinases expressed predominantly in T cells, and plays a pivotal role in T cell activation and differentiation (2). Through interaction with CD4 and CD8 co-receptor glycoproteins, Lck initiates signal transduction following engagement of the TCR complex (1, 3). Mice deficient in Lck are markedly depleted of T cells, and those T cells that do develop show a blunted response to TCR stimulation (4). Lck has also been shown to bind to the IL-2 receptor complex in T cells and participate in IL-2-mediated signaling (5).

Protein kinase D (PKD) is a family of serine/threonine kinases that belong to a subfamily of the calcium-calmodulin kinase-like superfamily. Three members of the PKD family have been identified so far: PKD1 (PKCμ) (6, 7), PKD2 (8) and PKD3 (PKCν) (9). The PKDs are implicated in the regulation of fundamental biological processes including signal transduction, membrane trafficking, cell survival, migration, differentiation and proliferation (10). The salient features of PKD structure include a tandem repeat of zinc-finger-like cysteine-rich motifs (referred to as cys1 or C1a, and cys2 or C1b, respectively) that are involved in recruiting PKD to the membrane, a pleckstrin-homology (PH) domain that plays an inhibitory role in the regulation of catalytic activity, and a C-terminal catalytic domain (11-14). Although PKD1, 2 and 3 share a similar modular structure, they have overlapping and yet distinct functional roles in various tissues and cell types (15-17). PKDs are activated by numerous stimuli including reactive oxygen species (ROS), growth factors (i.e. PDGF), activators of G-protein-coupled receptors and triggering of immune-cell receptors such as the B-cell receptor (BCR) or TCR complexes (15, 18-24). PKD1 is the most extensively characterized member among the three PKD isoforms, with much less being known about the regulation and biological roles of the other members of the PKD family. Similar to PKD1, PKD2 is activated by nPKCs (8, 25) and undergoes reversible translocation from the cytosol to the plasma membrane in response to GPCR stimulation (17). In contrast to PKD1, PKD2 is largely detectable in the cytoplasm after dissociation from the plasma membrane.

A recent study reports that PKD2 is predominantly expressed in T cells and contributes to IL-2 promoter regulation upon TCR stimulation (26). To further elucidate the regulation and biological functions of PKD2, we performed a yeast two-hybrid screen to identify novel PKD2 interacting partners. In this study, we demonstrated that Lck interacts with PKD2 and increases its activity. We also showed that PKD2 regulates NFAT activity upon TCR stimulation in Jurkat T cells.

RESULTS

PKD2 is associated with Lck

To identify proteins that interact with PKD2, a yeast two-hybrid screen of a human lymphoid cDNA library was performed using full-length PKD2 as the bait. From a total of $7.5 \times 10^5$
PKD2 interacts with Lck and regulates NFAT activity in T cells
Qing Li, et al.

colonies screened, Lck was identified as one of the five positive clones. To determine whether Lck and PKD2 associated with each other in vivo, 293T cells were co-transfected with Myc-Lck and Flag-PKD2 expression plasmids. Myc-Lck was immunoprecipitated from whole cell lysates with the anti-Myc antibody and Flag-PKD2 was readily detected in the precipitates, indicating that both proteins interacted with each other in mammalian cells (Fig. 1A).

A reciprocal co-immunoprecipitation experiment further confirmed the interaction between PKD2 and Lck (Fig. 1B). Interestingly, PKD1 also co-immunoprecipitated with Lck, although at a much lower level (Fig. 1B). To examine whether the observed interaction could occur at the endogenous level, lysates of Jurkat T cells were immunoprecipitated with the anti-Lck antibody or the control IgG. The precipitates were blotted with the anti-PKD2 antibody to detect the presence of PKD2. As shown in Fig. 1C, PKD2 was only detected from the precipitates obtained with anti-Lck antibody and not from the precipitates obtained with control IgG, indicating that endogenous PKD2 and Lck interacted with each other in T cells. Taken together, these data confirmed that PKD2 interacted with Lck in T cells.

Mapping the interacting domains in PKD2 and Lck
PKD2 contains N-terminal regulatory domains and a C-terminal kinase domain. To map out the domains on PKD2 that are essential for Lck interaction, we generated six truncated constructs of PKD2: PKD2 (261-878), PKD2 (1-390), PKD2 (1-549), PKD2 (1-260), PKD2 (390-878) and PKD2 (550-878). These constructs were co-transfected with Lck into 293T cells to examine their interaction with Lck by co-immunoprecipitation. The results showed that the C-terminal kinase domain of PKD2 (550-878) was both sufficient and necessary for its interaction with Lck (Fig. 2A). Lck also contains N-terminal regulatory do-

Fig. 1. PKD2 interacts with Lck in mammalian cells. (A) PKD2 associates with Lck in 293T cells. 293T cells were cotransfected with indicated plasmids. Cell lysates were immunoprecipitated with the anti-Myc antibody followed by immunoblotting with either anti-Flag or anti-Myc-HRP. (B) PKD2 interacts with Lck more strongly than PKD1. 293T cells were transiently transfected with indicated plasmids. Cell lysates were immunoprecipitated with the anti-Flag antibody followed by immunoblotting with indicated antibody. (C) Endogenous interaction between PKD2 and Lck. Jurkat T cell lysates were immunoprecipitated with the anti-Lck antibody and the precipitates were blotted with the anti-PKD2 antibody or anti-Lck antibody. Mouse IgG served as a control. WCL: whole cell lysates.

Fig. 2. Mapping the interacting domains in PKD2 and Lck. (A) Mapping the Lck interacting regions in PKD2. Top, 293T cells were transfected with indicated plasmids. Cell lysates were immunoprecipitated with the anti-Flag antibody followed by immunoblotting with the anti-HA antibody. Bottom, schematic representation of PKD2 fragments. (B) Mapping the PKD2-interacting regions in Lck. Top, 293T cells were transfected with indicated plasmids. Cell lysates were immunoprecipitated with the anti-HA antibody and blotted with either anti-HA-HRP or anti-Flag-HRP. Bottom, schematic representation of Lck fragments. WCL: whole cell lysates.
PKD2 interacts with Lck and regulates NFAT activity in T cells

Qing Li, et al.

Fig. 3. Lck enhances PKD2 tyrosine phosphorylation and increases its kinase activity. (A) 293T cells were co-transfected with Flag-PKD2 and either empty vector or Myc-Lck. Twenty-four hours later, the cells were pretreated with Lck inhibitor (50 nM PP2) for 6 h or not. Flag-PKD2 was immunoprecipitated from cell lysates using the anti-Flag antibody and its tyrosine phosphorylation level was analyzed by anti-P-Tyr immunoblotting. As shown in Fig. 3A, the tyrosine phosphorylation level of PKD2 was much higher when Lck was co-expressed, compared to the control vector, suggesting that Lck promoted PKD2 tyrosine phosphorylation. It was interesting that the Lck kinase inhibitor PP2 suppressed PKD2 tyrosine phosphorylation. To examine whether PKD2 kinase activity was enhanced by Lck, Flag-PKD2 was co-transfected into 293T cells along with the control vector, the vector expressing wild type Myc-Lck or the kinase-dead Lck. PKD2 was immunoprecipitated from cell lysates using the anti-Flag antibody and its kinase activity was measured by either auto-phosphorylation or phosphorylation of a common kinase substrate MBP. The results showed that the kinase activity of PKD2 was significantly enhanced when wild type Lck was co-expressed, compared to the vector control or kinase-dead Lck. These results demonstrated that Lck increased the kinase activity of PKD2 (Fig. 3B). To rule out the possibility that the enhanced phosphorylation of MBP was due to other unidentified kinase activity in the PKD2 precipitates, a kinase dead (KD) mutant of PKD2 was used as the control. Flag-PKD2 (KD) exhibited little catalytic activity on MBP. The amount of Lck present in the immune complexes was also investigated. The results showed that Lck associated equally with wild type or kinase dead PKD2 (Fig. 3B). Therefore, we confirmed that co-expression of Lck enhanced the kinase activity of PKD2.

PKD2 regulates NFAT activation induced by anti-TCR in T cells

Engagement of TCR leads to AP-1 activation in T cells, a critical pre-requisite for T cell activation, cytokine secretion and proliferation (1). Lck was shown to play critical roles in mediating TCR signaling and T cell activation. To investigate the physiological relevance of PKD2-Lck interaction, we examined the effects of over-expression of PKD2 on the activity of AP-1 transcription factor in Jurkat T cells. Our results showed that over-expression of PKD2 alone had no effect on AP-1 activity. However, over-expression of PKD2 enhanced anti-TCR-induced AP-1 activation significantly (Fig. 4A). This effect was dependent on the kinase activity of PKD2 because the kinase-dead mutant of PKD2
PKD2 interacts with Lck and regulates NFAT activity in T cells
Qing Li, et al.

Fig. 4. PKD2 enhances TCR-induced AP-1 and NFAT reporter activities in Jurkat T cells. (A) Jurkat T cells were transiently transfected with either pEF-Flag vector, pEF-Flag-PKD2 or pEF-Flag-PKD2m (kinase dead mutant) together with an AP-1 luciferase reporter plasmid. Cells were left untreated or treated with 1 μg/ml anti-TCR (C305) for 8 h. Luciferase activity in the cell lysates was determined in triplicates. The bottom panel shows the expression of transfected PKD2 determined by immunoblotting with anti-Flag. (B) Jurkat T cells were cotransfected with the indicated plasmids together with an NFAT luciferase reporter plasmid. Luciferase activity was determined as in (A). The bottom panel shows the expression of transfected PKD2. These data are representative of at least two independent experiments. Error bars represent standard errors of the means.

failed to increase anti-TCR induced AP-1 activation.

The nuclear factor of activated T cell (NFAT) family of transcription factors play important roles in the control of gene expression during cell activation and differentiation (29). To further study the roles that PKD2 plays in the TCR signaling pathway, we also examined the effects of overexpression of PKD2 on the activity of the NFAT transcription factor in Jurkat T cells. Similar to what was observed with the AP-1 reporter, the results showed that PKD2 by itself had no effect on NFAT activity. However, PKD2 enhanced TCR-induced NFAT activation significantly (Fig. 4B). In contrast, overexpression of PKD2 kinase dead mutant reduced the TCR-induced activation of NFAT. Taken together, these results suggested that PKD2 played a role in TCR-induced NFAT activation.

DISCUSSION

The PKD family of kinases shares similarities in domain structure, primary sequence and enzymological properties. But interesting differences in their expression pattern and subcellular distribution are also emerging (30). A recent study demonstrates that the major molecular species of the PKD family expressed in human peripheral T cells, mouse thymocytes and spleen cells is not PKD1, but PKD2 (26). We identified that PKD2 associated with Lck via the C-terminal kinase domains. Interestingly, we also observed an association between PKD1 and Lck but our results showed that PKD2 interacted with Lck more strongly than PKD1. This indicates that although PKD1 and PKD2 share a high homology within their N-terminal regulatory domains and C-terminal catalytic domain, PKD2 is a more important player in Lck signaling pathways.

PKD2 is known to be phosphorylated on both serine/threonine and tyrosine residues. nPKCs phosphorylate the two serine residues (Ser706 and Ser710) in the PKD2 phosphorylation loop leading to its activation (25). PKD2 is phosphorylated in the PH domain on Tyr438 by Bcr-Abl in myeloid leukemia cells, a site corresponding to Tyr463 in PKD1 (28). In this study, Lck increased the tyrosine phosphorylation of PKD2 as well as its catalytic activity. To test whether Lck also phosphorylated Tyr438 on PKD2, we constructed a Y438F mutant and co-transfected it with the Lck expression vector into 293T cells. However, tyrosine phosphorylation of the Y438F mutant exhibited no difference from that of the wild type PKD2 (data not shown), suggesting that tyrosine residues other than Tyr438 on PKD2 may be phosphorylated by Lck. A sequence analysis showed that potential tyrosine phosphorylation sites do exist on PKD2. Future studies are needed to map out the tyrosine residues phosphorylated by Lck.

The importance of PKD2 in lymphocyte biology has been elucidated in recent literature. Atsushi’s study showed that PKD2 is predominant in T cells and contributes to IL-2 promoter regulation upon TCR stimulation in Jurkat cells (26). Our results demonstrate that PKD2 can regulate TCR-induced NFAT and AP-1 activity in Jurkat T cells. More importantly, we showed that PKD2 interacts with Lck tyrosine kinase, a key regulator of TCR signaling. This corresponded to Atsushi’s study, which revealed that the PKD2 kinase activity could be upregulated by TCR stimulation regardless of the presence of ZAP-70 in Jurkat cells. It is possible that signals derived from TCR lead to PKD2 phosphorylation and activation directly by Lck, which in turn contributes to the regulation of NFAT activation and subsequently T cell activation.

Recent work has also shown that PKD2 can regulate gene transcription by phosphorylating the SET protein, a histone chaperone that modulates histone acetylation (26). Another study showed that PKD1 and PKD2 interact with and phosphorylates...
HDAC7, a member of the class Ila family of histone deacetylases, leading to its nuclear export (31). Nuclear HDAC7 can interact with transcription factor MEF2 and cause transcriptional repression of MEF2-targeted genes. Therefore, HDAC7 phosphorylation by PKD2 could lead to the transcriptional activation of certain genes. Further studies have to be conducted to determine whether the regulation of TCR-induced NFAT activation by PKD2 is through the phosphorylation of SET and/or HDAC7.

In summary, our findings demonstrate that the Src family of protein tyrosine kinase Lck associates with PKD2 and regulates its activity. The finding that PKD2 regulates NFAT activity induced by TCR in T cells indicates that PKD2 might be a new player involved in the regulation of T cell activation.

MATERIALS AND METHODS

Yeast two-hybrid screen
A yeast two-hybrid screen was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA, USA). Full-length human PKD2 (Gene Bank, NM_016457 cDNA was subcloned into the pGBKT7 vector and the resulting bait plasmid was used to screen a pACT2 human lymphoid cDNA library according to the manufacturer’s protocol. To eliminate false-positive clones, colonies were assayed for β-galactosidase activity using X-gal as the substrate.

Plasmids and antibodies
Full-length PKD1, PKD2 and PKD2 fragments were subcloned into the pEF expression vector with an N-terminal Flag tag. Full-length Lck and Lck fragments were subcloned into the pCDEF expression vector with an N-terminal Myc or HA tag. A PKD2 kinase dead mutant (PKDm, Lys580 to Ala) and an Lck kinase dead mutant (Lckm, Lys273 to Ala) were generated by site-directed mutagenesis using the QuikChange kit (Stratagene). Desired mutations were confirmed by sequencing. AP-1 and NFAT luciferase reporter constructs were kind gifts from Dr. Arthur Weiss (University of California, San Francisco, CA, USA).

Anti-Lck, anti-phospho-tyrosine (4G10) monoclonal antibodies and anti-TCR antibody (C305) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-PKD2 (BL843) polyclonal antibody was purchased from Bethyl Laboratories, INC. (Montgomery, TX, USA). Goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies conjugated with HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG (light chain specific) secondary antibodies conjugated with HRP was obtained from Jackson ImmunoResearch (West Grove, PA, USA). Anti-Flag, anti-Myc and anti-HA monoclonal antibodies were generated in-house. Flag, Myc, and HA antibodies conjugated with HRP (Flag-HRP, Myc-HRP, HA-HRP) were obtained from Sigma (St. Louis, MO, USA). The Lck kinase inhibitor PP2 was purchased from Calbiochem (San Diego, CA, USA).

Cell culture and transfection
Human embryonic kidney (HEK) 293T cells (ATCC: CRL-11268) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Gaithersburg, MD, USA) containing 10% FBS (GIBCO, Gaithersburg, MD, USA). Cells were transfected with indicated plasmids using calcium phosphate. Simian virus 40 T anti-gentransfected human leukemic Jurkat cells (Jurkat T, ATCC: TIB-152) were grown in RPMI 1640 medium (PAA) supplemented with 10% FBS (GIBCO, Gaithersburg, MD, USA). Cells were transfected with the indicated plasmids using the Superfect transfection reagent (Qiagen, Valencia, CA, USA).

Coimmunoprecipitation and Western blot analysis
293T cells transfected with indicated plasmids were harvested and lysed in lysis buffer as previously described (32). Lysates were subjected to immunoprecipitation with indicated antibodies. For Western blotting, the immune complexes were resolved by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and electrophoretically transferred. The membrane was incubated with the indicated primary antibody followed by secondary antibody incubation and assayed by enhanced chemiluminescence (SuperSignal Western Blotting Kit, PIERCE, Rockford, IL, USA).

In vitro kinase assay
To measure the kinase activity of PKD2, a kinase assay was performed as previously described (32).

Luciferase reporter assay
Approximately 1 × 10⁶ Jurkat T cells were transfected with 1 μg of AP-1 or NFAT luciferase plasmid together with 2 μg of indicated plasmids by Superfect transfection reagent (Qiagen, Valencia, CA, USA). Cells were cultured for 40 h after transfection and aliquoted into a 96-well plate (Corning, Acton, MA, USA) at 1 × 10⁵ cells per well. Cells were either untreated or stimulated with 1 μg/ml anti-TCR hybridoma supernatant at 37°C. After stimulation for 8 h, the cells were lysed in 25 μl of 5 × reporter lysis buffer (Promega, Madison, WI, USA) and the luciferase activity was assayed with a Lumat LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN, USA).

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PKD2 interacts with Lck and regulates NFAT activity in T cells
Qing Li, et al.

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