Differential Requirement for RhoA GTPase Depending on the Cellular Localization of Protein Kinase D*

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This study explores the links between the GTPase RhoA and the serine kinase protein kinase D (PKD) during thymocyte development. The rationale is that RhoA and PKD regulate common biological responses during T cell development, but there is nothing known about their interdependence. In fibroblasts, Rho function is required for activation of PKD catalytic activity. However, the data show that activation of Rho is neither sufficient nor essential for PKD activation in T cells. One alternative explanation for the apparent convergence of PKD and Rho signaling in T cells is that PKD responses might be Rho-dependent. To address this latter possibility, we probed the Rho requirements for the actions of constitutively active PKD mutants in pre-T cells of transgenic mice. Here we show that membrane-localized PKD regulation of pre-T cell differentiation is Rho-dependent, but the actions of cytosol-localized PKD are not. These studies demonstrate that a Rho requirement for PKD activation is not ubiquitous. Moreover, links between PKD and Rho are determined by the cellular location of PKD.

Protein kinase D1 is a member of a conserved family of serine/threonine kinases that includes PKD2 and PKCα/PKD3 (1, 2). PKD1 contains multiple domains with the N-terminal region composed of two cysteine-rich motifs, which display high affinity for diacylglycerol (DAG)4 or tumor-promoting phorbol esters and a pleckstrin homology domain (3, 4). The C-terminal region contains the serine/threonine catalytic kinase domain that shares homology with the calmodulin-dependent kinases. PKDs are expressed ubiquitously and can be activated by a wide variety of physiological stimuli, including growth factors, neuropeptides, and oxidative stress (4). PKDs are particularly abundant in lymphocytes where they are selectively activated by triggering of antigen receptors (5, 6). The activation of PKD in lymphocytes requires DAG binding and protein kinase C (PKC)-mediated phosphorylation of two serine residues (Ser-744 and 748) within the activation loop of the catalytic domain (1, 6).

Recently, it has been shown that PKD has the capacity to regulate the functional activity of β1 integrins via regulation of the Rap1 GTPase (7). As well, a recently proposed function for PKD in lymphocytes is the phosphorylation of class II histone deacetylases (8–10). The activation of PKD by antigen receptors is a sustained response and is also associated with changes in PKD intracellular location (11). In the initial phase of lymphocyte activation there is a rapid translocation of PKD to the plasma membrane. However, during sustained activation, PKD relocates to the cytosol where it remains active for a prolonged period of time (11). The function of PKD at these different locations has been probed in an in vivo model using active PKD mutants targeted to either the plasma membrane or the cytosol of pre-T cells of transgenic mice. Studies of these mice have shown that PKD can substitute for the pre-T cell receptor and induce both proliferation and differentiation of T cell progenitors in the thymus. Moreover, cellular localization of PKD within a thymocyte is critical; membrane-targeted and cytosolic PKD thus control different facets of pre-T cell differentiation (12). The idea that PKD function is determined by its intracellular localization is supported by more recent studies showing that Golgi-localized PKD regulates phosphatidylinositol 4-kinase IIIβ, whereas PKD localized to the mitochondria controls NF-κB-induced expression of SOD2, a gene involved in apoptosis (13, 14).

PKDs are substrates for PKCs (15), but other than this connection little is known about where PKD is positioned relative to other signal transduction pathways. A number of recent studies have shown links between PKD and Rho GTPases. Hence, a genetic screen for signaling pathways involved in regulating the class II histone deacetylases in COS cells not only identified PKD2 but also RhoA, RhoC, and the guanine nucleotide exchange factors RhoGEF1 and RhoGEF5 as histone deacetylase regulators (16). PKD activation has also been shown to be required for secretion of a gut peptide, neuropeptin, from endocrine cells, and critical upstream regulators include PKCα, PKCδ, and the Rho/ROK pathway (17). Moreover, activation of phospholipase C-ε by heterotrimeric G proteins, Go12/13 requires a direct interaction of RhoA with phospholipase C-ε, implicating RhoA function in lipid metabolism.

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‡1 The abbreviations used are: DAG, diacylglycerol; FACS, fluorescence-activated cell sorter; PKD, protein kinase D; PKC, protein kinase C; PBS, phosphate-buffered saline; TCR, T cell receptor; PE, phycoerythrin; SRE, serum-response element; GFP, green fluorescent protein.
Rho and PKD

(18). The simplest model for Rho involvement in PKD signal transduction has been proposed in fibroblasts where bombesin stimulation via G13 heterotrimeric proteins activates PKD in a PKC-dependent pathway, which also requires Rho function (17, 19, 20).

The relationship between PKD and RhoA in T cells has not been examined directly, but there is circumstantial evidence for a connection. For example, both PKD and RhoA are involved in the Rap1A-mediated pathways that regulate β1 integrins (7, 21). It is also known that RhoA, like PKD, can control pre-T cell differentiation (22). Hence, the loss of Rho function severely impairs T cell development and prevents pre-T cell receptor and p56 lck-induced thymocyte differentiation (23, 24). One explanation for the shared ability of PKD and RhoA to control T cell responses is that as in fibroblasts, RhoA is required for PKD activation in T cells. In this respect, in B lymphocytes RhoA regulates antigen receptor-induced production and hydrolysis of phosphatidylinositol 4,5-bisphosphate, a key step for the production of DAG that mediates PKD activation (25). However, whether Rho regulation of PKD activation occurs in T cells is not known.

In this study the objective is to explore the involvement of Rho in PKD signal transduction in T cells because both PKD and RhoA are important signal transduction molecules in T cells, but it is not known whether they work independently. The present results found that RhoA activation is not sufficient or essential for PKD activation in T lymphocytes. There is a requirement for RhoA function for T cell responses triggered by PKD, but the PKD dependence on Rho is determined by the cellular location of this serine kinase. Links between PKD and Rho signal transduction thus differ depending on cell lineage, cell stimulus, and intracellular location of PKD.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Vectors encoding a chimeric fusion protein between green fluorescent protein (GFP) and wild type PKD1 or the constitutively active membrane-targeted PKD have been described before (6, 12, 26). pEF-link vectors expressing N-terminal 9E10 epitope-tagged C3 transferase or V14RhoA have been described previously (27).

Cell Preparation and Stimulation—The human T lymphoma line Jurkat 6.2 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Transient transfection of Jurkat was carried out by electroporation at 310 V, 950 μF using a 4-mm cuvette (Bio-Rad Gene Pulsar Xcell). Cells were allowed to recover overnight before experimental use. Cell stimulation was carried out using medium alone, 10 μg/ml phorbol 12,13-dibutyrate for 10 min at 37 °C. Stimulated cells were washed in ice-cold phosphate-buffered saline and subjected to Western blot analysis.

SRE Reporter Gene Assays—Chloramphenicol acetyltransferase (CAT) assays were carried out essentially as described (28, 29). A TKCAT reporter plasmid controlled by two copies of the serum-response element (SRE) was described previously (30). Briefly, Jurkat cells were transfected as described above with an SRE-CAT reporter gene comprising V14RhoA (2 μg) alone or with C3 transferase constructs (5 μg). Cells were collected after 24 h, washed with PBS, and analyzed for CAT activity. Briefly, cells were lysed in 150 μl of lysis buffer (0.65% Nonidet P-40, 10 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl) for 10 min on ice. Cell debris was removed by centrifugation, and the lysate was heat-treated at 68 °C for 10 min before use. Lysates were incubated in a solution containing 150 μM Tris, pH 8.0, 0.05 μCi of [14C]chloramphenicol, and 2 μM acetyl coenzyme A for 24 h. Chloramphenicol was extracted with ice-cold ethyl acetate, and the amount of radioactivity in the acetylated products and nonacetylated substrate was determined by liquid scintillation counting of organic and aqueous phases, respectively. Results are expressed as percentage conversion of chloramphenicol to the acetylated form.

Cell Lysis and Western Blot Analysis—Cells were lysed for 15 min at 4 °C (20 × 10⁶ cells/ml) using 100 mM Hepes, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM sodium fluoride, 20 mM iodoacetamide, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml antipain, 40 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. Soluble proteins were concentrated by precipitation with 1.5 volumes of acetone and incubated at −20 °C for at least 1 h. Protein concentrates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by Western blotting analysis with the appropriate antibodies. Phospho-Ser-916 PKD antibody has been described previously and was used to detect PKD activity (26). PKD phospho-Ser-744/748 antibody was purchased from Cell Signaling Technology. Monoclonal GFP antibody was used in Western blot analysis to verify expression levels. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Transgenic Mice—Mice were bred and maintained under specific pathogen-free conditions in the transgenic animal unit. Transgenic mice expressing the constitutively active membrane-targeted PKD or cytosolic targeted PKD in the thymus under the control of CD2 promoter and locus control region have been described previously (12). C3 transgenic mice, which selectively express the bacterial toxin C3 transferase under the control of CD2 promoter and locus control region in the thymus, have been described in detail elsewhere (24).

Flow Cytometric Analysis—Fluorescein isothiocyanate, phycoerythrin (PE), allophycocyanin, and biotin-conjugated antibodies were obtained from Pharmingen. Tricolor-conjugated antibodies or streptavidin antibodies were from Caltag (Burlingame, CA). Cells were stained for cell surface markers and analyzed on a FACScalibur fluorescence-activated cell sorter (FACS; BD Biosciences). Data were analyzed using CellQuest software. CD4–CD8– double negative T cell subsets were analyzed for CD44 and CD25 expression by lineage exclusion of mature DP and SP cells as well as non-T cell lineage cells using a mixture of biotinylated antibodies (CD4, CD8, CD3 B220, Mac-1, NK, Gr-1, and γδ) revealed with streptavidin tricolor and costained with CD25-FITC, CD44-PE, and Thy1.2-APC. Surface expression of membrane-targeted PKD was assessed using the OX34 monoclonal antibody as described (12).

Intracellular TCRβ staining was carried out on thymocyte subpopulations by antibody staining, which were subsequently fixed in 1% paraformaldehyde for 10 min at room temperature, washed in PBS, and permeabilized with saponin buffer (0.5% saponin, 5%
FBS, 10 mM Hepes, pH 7.4, in PBS) for 10 min at room temperature. Permeabilized cells were incubated with PE-conjugated TCRβ antibody for 45 min at room temperature in saponin buffer, washed in saponin buffer, and analyzed by FACS. Cell surface binding sites were blocked using biotinylated TCRβ antibody, and specificity of staining was verified by parallel staining with PE-conjugated isotype-matched control antibody (Armenian hamster IgG group 2A).

RESULTS

PKD Activation Does Not Require Endogenous RhoA Function—The activity of RhoA is required for PKD activation in fibroblasts and intestinal cells (19) (17, 20). To investigate the involvement of endogenous RhoA in PKD activation in lymphocytes, the effects of expressing *Clostridium botulinum* C3 transferase, which selectively inhibits RhoA function, on antigen receptor activation of PKD in T cells was examined. *C. botulinum* C3 transferase ADP-ribosylates Asn-41 on RhoA, thereby preventing its ability to regulate downstream targets. The data in Fig. 1A show the impact of C3 transferase expression on PKD activation induced by T cell antigen receptor ligation or following exposure to the phorbol ester, phorbol 12,13-dibutyrate, a pharmacological mimic of DAG. PKD activation is mediated by phosphorylation of residues Ser-744 and Ser-748 in the catalytic domain of the enzyme, and this results in immediate autophosphorylation of PKD1 on a C-terminal autophosphorylation site, Ser-916. Accordingly, PKD1 catalytic activity “*in vivo*” (i.e. in an intact cell) can be monitored using phospho-specific antisera that selectively recognize PKD molecules phosphorylated on Ser-744/748 or Ser-916.

The data (Fig. 1A) show that both phorbol ester and antigen receptor activation of PKD in lymphocytes is accompanied by phosphorylation of Ser-744/Ser-748 and Ser-916 antibodies to monitor PKD activity or with a GFP monoclonal antibody to reveal total PKD levels.
blocked by expression of C3 transferase indicating that TCR-induced activation of PKD can occur in the absence of endogenous Rho function. ADP-ribosylated RhoA has reduced electrophoretic mobility in SDS-PAGE. The efficacy of C3 transferase in terms of RhoA ribosylation was thus confirmed by Western blot analysis of RhoA (Fig. 1B). Additionally, C3 transferase could block the transcriptional activation of serum-response factor (SRF) by expression of constitutively active V14RhoA in T cells (Fig. 1C).

In COS7 cells expression of active mutants of RhoA can stimulate basal activity of PKD (19). Accordingly, we examined the effect of expressing V14RhoA, the constitutively active mutant of RhoA, on PKD activation in T cells. The data (Fig. 1D) show that V14RhoA did not stimulate the basal activity of PKD in T cells nor did it synergize with antigen receptor stimulation of phorbol esters for PKD activation. The positive control for V14RhoA functional activity is shown in Fig. 1B; these data show expression of V14RhoA induced a strong activation of SRF.

The Role of RhoA for Functional Responses Induced by PKD in T Cells—RhoA does not appear to be required for PKD activation in T cells, but we considered an alternative possibility that RhoA signaling pathways might mediate PKD actions. We have recently developed transgenic mouse models with gain of function mutants of PKD targeted to the plasma membrane or cytosol of pre-T cells that allow us to explore PKD-mediated responses in pre-T cells in vivo. We also have available transgenic mice that express C. botulinum C3 transferase (C3 mice) under the control of T cell-specific promoters in pre-T cells. Accordingly, by generating mice doubly transgenic for PKD mutants and C3 transferase, we can assess whether biological responses induced by PKD activation at the membrane or cytosol of pre-T cells requires endogenous RhoA function.

During thymocyte development the pre-T cell receptor, which activates PKD, controls a critical differentiation checkpoint known as β-selection (31, 32). T cell progenitors that enter the thymus from the bone marrow lack expression of the major histocompatibility coreceptors CD4 and CD8 and are termed double negatives (CD4− and CD8−). The commitment of these progenitors to the T cell lineage begins with rearrangements of the TCR-β locus that, if successful, results in expression of the β-subunit of the antigen receptor as part of the pre-T cell receptor complex. The pre-TCR then instructs cells to proliferate rapidly and induces a well characterized set of genetic
changes that control T cell differentiation. The pre-TCR-induced genetic program includes modulation of the cell membrane phenotype of pre-T cells. The pre-TCR thus switches off expression of CD25, the interleukin-2 receptor α subunit, but up-regulates expression of other cell surface receptors, including the coreceptors CD4 and CD8, the adhesion molecule CD2, and signaling molecules such as CD5 (33).

In the absence of a pre-TCR T cells become blocked in differentiation at a CD25+ CD2− CD5− CD4− CD8− stage; for example in recombinase activating gene 2 (RAG2−/−), null mice which cannot undergo TCR gene rearrangements (34, 35). One role for the pre-TCR is to activate PKD, and accordingly, expression of active PKD mutants can bypass the absence of the pre-TCR and induce pre-T cell differentiation (12). Triggering of antigen receptors causes a transient relocalization of PKD to the lymphocyte plasma membrane, and to probe the function of PKD at the membrane, transgenic mice expressing a mutated active membrane-targeted PKD under the control of T cell-specific promoters have been produced (membPKD) (12). To target active PKD to the plasma membrane, the catalytic core of PKD was fused to the extracellular and transmembrane domain of the cell surface receptor rat CD2 (rCD2) to create a chimera (membPKD). The characterization of the membPKD chimera has been described (12).

The backcrossing of membPKD mice onto a RAG2−/− genetic background, where there is no endogenous pre-TCR and no activation of endogenous PKD, makes it possible to assess the functional consequence of activating PKD at the plasma membrane in pre-T cells in the absence of any other pre-TCR signaling pathways. The data in Fig. 2A show that expression of active PKD at the plasma membrane of RAG2−/− null mice down-regulates expression of CD25 but up-regulates expression of CD4, CD8, and CD5. The activity and plasma membrane localization of the CD2-membPKD chimera is not inhibited when RhoA function is inhibited by expression of C. botulinum C3 transferase (Fig. 2, B and C). Hence to investigate whether membPKD-mediated signaling in pre-T cells requires endogenous RhoA function, we expressed active membrane-targeted PKD in pre-T cells that lack RhoA function because of expression of C3 transferase. This was achieved by breeding transgenic mice expressing active membrane-targeted PKD under the control of the CD2 promoter with transgenic mice expressing C. botulinum C3 transferase (Fig. 2, B and C). Hence to investigate whether membPKD-mediated signaling in pre-T cells requires endogenous RhoA function, we expressed active membrane-targeted PKD in pre-T cells that lack RhoA function because of expression of C3 transferase. This was achieved by breeding transgenic mice expressing active membrane-targeted PKD under the control of the CD2 promoter with transgenic mice expressing C. botulinum C3 transferase also under the control of the CD2 promoter (C3 mice). Previous studies have shown that inhibition of RhoA function in C3 mice prevents β-selection and causes T cells to become blocked in differentiation at a CD25+ CD2−CD5−CD4−CD8− stage indistinguishable from the phenotype of RAG2−/− mice (24). C3 transferase can ADP-ribosylate different members of the Rho GTPase family, but microarray analysis of pre-T cell subsets using the Affymetrix Mouse Genome 430 2.0 array has shown that T cell progenitors express RhoA but not RhoB or RhoC (data not shown). Accordingly, the ability of C3 transferase to block pre-T cell development reflects a requirement for the function of RhoA for β-selection. The data in Fig. 2D show that membrane-targeted PKD cannot induce CD25 down-regulation in pre-T cells that express C3 transferase nor can this active PKD mutant up-regulate expression of CD4 and CD8 or CD5. Membrane-targeted PKD can thus bypass loss of pre-TCR function in pre-T cells in the RAG2−/− mice but cannot bypass loss of RhoA function in C3 transgenic mice.

Cytosol-targeted Active PKD-mediated T Cell Responses Are RhoA-independent—The requirement for RhoA function for the actions of membrane-targeted PKD is in accordance with a role for RhoA in the transmission of signals from the plasma membrane to the cell interior. In this context, C3 transferase-mediated ADP-ribosylation of RhoA has been shown to block the functional activity of this GTPase by trapping it in a cytoplasmic protein complex and preventing its translocation to the plasma membrane where it interacts with its effectors (36). During sustained antigen receptor responses, PKD is active in the cytosol. To probe the function of PKD in the cytosol, we have used the CD2 transgenic promoter to make transgenic mice expressing a cytosol-localized constitutively active mutant of PKD in pre-T cells (cytoPKD). The activation of PKD in the cytosol has some common responses with the membrane-targeted PKD mutant. Hence in RAG2−/− pre-T cells cytoPKD down-regulates expression of CD25 and up-regulates expression of CD2 and CD5 (Fig. 3). However, the genetic changes induced by cytoPKD are not identical to those triggered by PKD at the membrane. There is thus a unique response to activating PKD in the cytosol, namely inhibition of TCR-β locus gene rearrangements, which results in the suppression of TCR-β chain expression in pre-T cells. Membrane-targeted PKD does not down-regulate TCR-β expression (12).

To probe the role of RhoA in the responses induced by cytosol-targeted PKD, C3 transgenic mice were bred with cytoPKD transgenic mice. The data in Fig. 4A show that the ability of cytosol-targeted PKD to down-regulate expression of CD25 is not blocked by expression of C3 transferase. Similarly, the ability of cytosol-targeted PKD to up-regulate expression of CD2 and CD5 (Fig. 4A) is not blocked by C3 transferase. The data in Fig. 4B compare TCR-β chain expression in wild type pre-T cells and pre-T cells that express active cytosolic PKD demonstrating that pre-T cells that express active cytosolic PKD lack expression of TCR-β subunits compared with wild type cells. The ability of cytoPKD to inhibit expression of TCR-β chains is independent of endogenous RhoA function, as thymocytes that coexpress active cytosolic PKD and C3 transferase also fail to express TCR-β chains (Fig. 4B).
Rho and PKD

One common response to both membrane- or cytosol-localized PKD is that in the absence of the pre-TCR in RAG2−/− mice, membrane-targeted or cytosolic PKD can induce a comparable proliferative expansion of pre-T cells (12). The data in Fig. 4C compare thymocyte numbers in C3 transgenic mice in the presence or absence of membrane- or cytosol-targeted active PKD. Cell numbers in C3 thymi range from 2.5 to 3 × 10⁶, and this does not change when membrane PKD is expressed. In contrast expression of cytosol-targeted PKD resulted in an ~3-fold increase in thymocyte numbers in C3 mice. Hence, cytosol-localized PKD can induce proliferative expansion of pre-T cells without RhoA function, whereas membrane-localized PKD cannot.

DISCUSSION

This study explores links between the GTPase RhoA and the serine kinase PKD in T cells. The rationale for this work is that RhoA and PKD are both key intracellular signaling molecules that regulate common biological responses during T cell development in the thymus, but how interdependent they are has not been explored. One objective was to determine whether Rho function is required for activation of PKD catalytic activity in T cells, as described for the regulation of PKD activity in fibroblasts (19). The data herein show that activation of RhoA is neither sufficient nor essential for PKD activation in T cells. Accordingly, the RhoA requirement for PKD activation is not ubiquitous but is dependent on cell type and stimulus. One alternative explanation for the apparent convergence of PKD and RhoA signaling in thymocytes is that PKD responses might be RhoA-dependent.

To address this latter possibility we probed the RhoA requirements for the actions of constitutively active PKD mutants in pre-T cells of transgenic mice. Two different PKD transgenic models were used as follows: one that expressed a membrane-targeted active PKD mutant, and one that expressed a cytosol-targeted PKD mutant. In both models, the human CD2 promoter and locus control region was used as transgenic vectors to switch on transgene expression in early pre-T cells. PKD transgenic mice were produced originally to compare the function of membrane- and cytosol-localized PKD. The motivation for this work was that in T lymphocytes PKD is activated by antigen receptors and is initially localized to the plasma membrane. However, during sustained responses PKD relocates to the cytosol where it remains active for several hours. It was therefore important to develop experimental models to probe PKD function at different intracellular locations.

Previous characterization of PKD transgenic mice showed that a change in the intracellular location of PKD changes the biological role of the protein (12). For example, the expression of the CD4 coreceptor in thymocytes that lack a pre-TCR (RAG2−/− thymocytes) is only induced by PKD localized to the plasma membrane and not by cytosolic PKD. As well, cytosolic active PKD suppresses TCRβ chain expression in pre-T cells, whereas membPKD does not. This study shows that these unique responses to membrane or cytosolic PKD differ in their sensitivity to RhoA inhibition. Hence membrane PKD up-regulation of CD4 and CD8 expression is RhoA-sensitive, whereas the ability of cytosolic PKD to prevent TCRβ chain expression is RhoA-independent. However, membrane-targeted and cytosolic active PKD can also trigger some common responses in T cell progenitors in the absence of the pre-TCR as follows: down-regulation of CD25, up-regulation of CD2 and CD5, and pre-T cell proliferation. Strikingly, this study shows that the shared actions of membrane and cytosolic PKD in pre-T cells can be distinguished by their differential requirements for RhoA. For example, membrane-targeted PKD needs RhoA function to down-regulate CD25 expression and up-regulate CD5 expression in pre-T cells, whereas cytosol-targeted PKD does not. As well, membrane PKD cannot induce proliferative expansion of pre-T cells lacking RhoA function, whereas cytosol-targeted PKD can. These results show that signaling links between PKD and RhoA are not universal but dictated by the cellular localization of the enzyme. Furthermore, even when membrane- and cytosol-localized PKD regulate common genetic changes, the difference in RhoA sensitivity indicates that membrane and cytosol PKD use divergent downstream signaling pathways.

Why does the function of PKD at the plasma membrane require RhoA function? In this respect there is no evidence that activation of membrane PKD results in RhoA activation (data

Figure 4. A, cytosol-targeted active PKD induces the expression of cell surface markers in pre-T cells in a Rho-independent manner. Histograms show CD25, CD2, and CD5 staining profiles in total Thy1.2+ thymocytes from CytoPKD and CytoPKD/C3 mice. B, cytosolic targeted active PKD inhibition of TCR-β chain expression is independent of endogenous Rho function. Histogram shows intracellular staining of TCR-β chains (filled box) in NLC, CytoPKD, and CytoPKD/C3 DNA cells. Isotype-matched control antibody shows negative staining (dotted line). C, CytoPKD but not MemPKD can induce a proliferative expansion in thymocytes that lack endogenous RhoA function. Data show total Thy1.2+ thymocytes in C3, MemPKD/C3, or CytoPKD/C3 mice (n = 3).
not shown), nor do constitutively active mutants of RhoA mimic the effects of active PKD mutants in pre-T cells (37). Hence, RhoA is not linked in a linear mode to membrane PKD but is more likely part of a parallel signaling pathway. In this respect, C3 transferase-mediated ADP-ribosylation of RhoA is shown to prevent membrane translocation of RhoA and thereby prevent interactions between this GTPase and its effectors (36, 38). The present results are consistent with this model and suggest that RhoA functions in the transmission of signals from the plasma membrane to the cell interior and not in the regulation of signaling that originates in the cytosol. One alternative hypothesis is that RhoA regulates the expression/localization of substrates for membrane-localized PKD. For example, RhoA function is needed for normal activation of β1 integrins in thymocytes (21), and in the absence of RhoA function, defective integrin activation/clustering may cause cytoskeletal disruption thereby preventing membrane-targeted PKD making contact with key substrates. Similarly, a well defined role for RhoA in many cells is the regulation of actin treadmilling, and changes in the actin cytoskeleton (39) may impact on the ability of membrane PKD to contact substrates. The positioning of PKD in the cytosol would physically distance the kinase from the membrane cortical actin structures that form the actin cytoskeleton in lymphocytes, hence making it insensitive to regulation by cytoskeletal modifications. To resolve these issues it will be necessary to identify PKD substrates at the plasma membrane and in the cytosol and subsequently assess the role of RhoA in regulating their expression and/or subcellular localization. However, to date PKD substrates at the plasma membrane are not yet known.

In conclusion, this study shows that in T cells RhoA is not needed for PKD activation but is required for pre-T cell differentiation induced by membrane but not cytosolic PKD. The observation that membrane-localized but not cytosol-localized PKD needs RhoA is intriguing because although PKD expression is ubiquitous, this enzyme has a different pattern of intracellular localization in different cell types. For example, in HeLa cells PKD is found in the trans-Golgi network and the mitochondria but not the plasma membrane (13, 14). Moreover, although shuttling of PKD between the cytosol and the plasma membrane occurs in a number of cell lineages, the kinetics of this response varies depending on cell type and stimulus (11, 40–43). Accordingly, the window of time for the RhoA dependence of PKD signal transduction will vary depending on the intracellular position of PKD, and this will differ depending on cell lineage and/or cell stimulus. The differential sensitivity of membrane- or cytosol-targeted PKD to RhoA inhibition reveals that the functional capabilities of this kinase are dictated by its intracellular location. There is thus no ‘hardwiring’ of PKD signal transduction, rather changes in the intracellular distribution of this kinase allow it to link to different signal transduction pathways.

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