

Dual Phospholipase C/Diacylglycerol Requirement for Protein Kinase D1 Activation in Lymphocytes*

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The serine/threonine kinase protein kinase D1 (PKD1) is a protein kinase C (PKC) substrate that mediates antigen receptor signal transduction in lymphocytes. PKC phosphorylates serines 744/748 within the PKD1 catalytic domain, and this is proposed to be necessary and sufficient for enzyme activation. Hence, a PKD1 mutant with alanine substituted at positions 744 and 748 (PKD-S744A/S748A) is catalytically inactive. Conversely, a PKD1 mutant with glutamic residues substituted at positions 744 and 748 as phospho-mimics (PKD-S744E/S748E) is constitutively active when expressed in Cos7 or HeLa cells. The present study reveals that Ser-744/Ser-748 phosphorylation is required for PKD1 activation in lymphocytes. However, PKD-S744E/S748E is not constitutively active but, like the wild type enzyme, requires antigen receptor triggering or phorbol ester stimulation. Antigen receptor activation of wild type PKD is dependent on phospholipase C (PLC)/diacylglycerol (DAG) and PKC, whereas PKD-S744E/S748E is only dependent on PLC/DAG but no longer requires PKC. Hence, substitution of serines 744 and 748 with glutamic residues as phospho-mimics bypasses the PKC requirement for PKD1 activation but does not bypass the need for antigen receptors, PLC, or DAG. In lymphocytes, PKD1 is, thus, not regulated by PLC and PKC in a linear pathway; rather, PKD1 activation has more stringent requirements for integration of dual PLC signals, one mediated by PKCs and one that is PKC-independent.

Protein kinase (PK)¹ D1 is a member of an evolutionarily conserved serine/threonine kinase family that in mammals has two other members, PKD2 and PKD3 (1–3). In fibroblasts and epithelial cells PKD1 is a component of the signaling pathways triggered by G protein-coupled receptor agonists, receptor tyrosine kinases, or oxidative stress (2, 4, 5). PKDs are highly expressed in lymphocytes and are strongly and selectively activated by the T cell antigen receptor, B cell receptor (BCR),

and FcεR1 in T cells, B cells, and mast cells, respectively (6–8). Antigen receptor ligation induces sustained PKD1 activation (6, 7). PKD expression is strongest in the thymus, where it is activated by the pre-T cell antigen receptor complex in T cell precursors (9). In these cells active PKD1 can substitute for the pre-T cell antigen receptor and induce substantial thymocyte proliferation and differentiation (9).

PKDs have an N-terminal cysteine-rich domain (CRD) that forms a high affinity binding site for diacylglycerol (DAG) or tumor-promoting phorbol esters (4, 10). DAG or phorbol ester binding to PKDs controls the intracellular localization of these enzymes and originally resulted in their designation as PKC isoforms. However, PKDs are structurally and catalytically distinct to PKCs with different substrate specificities and modes of regulation (2, 3, 11). In fact, the relationship between PKCs and PKD1 is a regulatory one, as in a variety of cell lineages stimulation of PKCs is necessary for PKD activation (6, 12, 13). The role of PKCs in PKD1 activation is explained by their ability to phosphorylate serines 744 and 748 within the PKD1 catalytic domain (13, 14). The phosphorylation of Ser-744 and Ser-748 is crucial for PKD1 activation in epithelial cells as substitution of these residues with alanines creates a catalytically dead kinase (15). Conversely, substitutions of acidic residues at Ser-744 and Ser-748 as a phospho-mimic has been shown to render PKD1 constitutively active in Cos7 and HeLa cells (15, 16).

The importance of PKCs for PKD1 activation explains why PKD1 is regulated by phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-diphosphate, producing DAG, thereby activating PKCs. Current models for PKD1 activation, thus, propose that the role of DAG or phorbol esters in PKD1 activation reflects DAG requirements for PKC activation. PKC activation of PKD1 in fibroblasts occurs at the plasma membrane and involves scaffolding proteins such as the protein kinase A-anchoring protein AKAP-Lbc (17, 18). DAG is also required for the colocalization of PKD1 and PKC to allow PKC-mediated transphosphorylation of PKD (17). It is proposed that the PKD N terminus maintains the molecule in an inactive conformation, which is relieved by phosphorylation of Ser-744 and Ser-748 by PKCs (14, 19). In this minimal model the phosphorylation of Ser-744/Ser-748 is sufficient for PKD1 stimulation (13, 20, 21). One basis for this conclusion is that a PKD1 mutant with glutamic residues substituted at positions 744 and 748 as phospho-mimics (PKD-S744E/S748E) is constitutively active in Cos7 and HeLa cells. PKD-S744E/S748E has, thus, been used as a “gain of function” mutant in a variety of studies that probe the functional role of PKD1 (16, 22, 23).

Current models for PKD1 regulation are based on experiments performed in fibroblasts or epithelial cells in the context of G protein-coupled receptor agonists, receptor-tyrosine

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¹ The abbreviations used are: PK, protein kinase; DAG, diacylglycerol; PDBu, phorbol 12,13-dibutyrate; PLC, phospholipase C; BCR, B cell receptor; PH, pleckstrin homology; CRD, cysteine-rich domain; GFP, green fluorescent protein.

kinases, or oxidative stress. However, PKD1 shows different patterns of intracellular localization in different cell types (24–26) that could influence its ability to respond to different stimuli. Indeed, the PKC isoform that mediates PKC activation can differ between different cell types; PKC δ controls PKD1 activation by oxidative stress in HeLa cells (16), whereas PKC ϵ is necessary for G protein-coupled receptor stimulation of PKD1 in Swiss 3T3 cells (17). There is also evidence for divergence in PKD1 regulation by different stimuli, as in HeLa cells the phosphorylation of tyrosine 463 was important for PKD1 activation in response to oxidative stress but not for PKD1 activation by a receptor-tyrosine kinase, the platelet derived growth factor receptor (27).

In lymphocytes the role of PKC-mediated phosphorylation of PKD1 has not been explored. In this context recent results argue that one cannot extrapolate data about PKD regulation in fibroblasts to lymphocytes. For example, G protein-coupled receptor activation of PKD1 in fibroblasts is dependent on plasma membrane translocation of the enzyme, whereas antigen receptor stimulation of PKD lymphocytes is not (6, 7, 17). Accordingly, the object of the present study was to explore the role of PKC-mediated phosphorylation of PKD1 in lymphocytes. The results reveal that PKD1 activation in lymphocytes has stringent requirements for integration of dual PLC signals; that is, one mediated by PKCs and one that is PKC-independent. The ability of PLC/DAG to regulate PKD1 independently of Ser-744/Ser-748 phosphorylation has not been observed previously and indicates that the extracellular signaling requirements for PKD1 differ dependent on the experimental model.

MATERIALS AND METHODS

cDNA Constructs—cDNA constructs encoding wild type PKD1 and various PKD1 mutants in the pcDNA3 mammalian expression vector have been described previously: PKD1 wild type, PKD Δ PH, PKD-S744A/S748A, PKD-S744E/S748E, and PKD-P155G/P287G (15, 19, 28, 29). cDNA constructs encoding a chimeric fusion protein between green fluorescent protein (GFP) and the above PKD1 mutants were generated by subcloning an EcoRI cDNA fragment encoding the PKD1 constructs into the EcoRI site of pEF-*plink2*-GFP_{C3} (7) to generate a GFP-PKD expression vector. Constructs were verified by DNA sequencing and purified by CsCl density gradient centrifugation before use in transient transfection experiments.

Cell Preparation and Stimulation—The BALB/c mouse B lymphoma line A20 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μ M β -mercaptoethanol. The human T lymphoma line Jurkat 6.2 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The chicken B lymphoma DT40 line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% chicken serum. Transient transfection of the A20 B cell lymphoma cell line has been described previously (7). The Jurkat human T-cell lymphoma line was transiently transfected by electroporation at 140 V, 1000 microfarads using a 2-mm cuvette (Bio-Rad Gene Pulser Xcell). Transient transfection of the DT40 chicken B cell lymphoma line as well as a DT40 PLC γ 2^{-/-} cell line (30) was carried out using the described previously A20 protocol, with the exception that the cuvettes were chilled on ice for 10 min both pre- and post-electroporation.

To inhibit the action of PKC in intact cells, lymphocytes were preincubated with medium alone or 5 μ M GF109203X for 20 min at 37 °C before cell stimulation. To monitor PKD1 activity in intact cells, lymphocytes were stimulated with medium alone or 20 ng/ml phorbol 12,13-dibutyrate for 10 min at 37 °C. Ligand of the A20 and DT40 BCR was carried out by adding, respectively, 10 μ g/ml rabbit anti-mouse F(ab')₂ fragment (Zymed Laboratories Inc.) (A20) or 20 μ g/ml anti-chicken IgM monoclonal antibody M4 (DT40) for 10 min at 37 °C. Unless otherwise indicated stimulated cells were washed in ice-cold phosphate-buffered saline and subjected to Western blot analysis.

Cell Lysis and Western Blot Analysis—Cells were lysed for 10 min at 4 °C (20 \times 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 μ M EDTA, 1 mM sodium orthovanadate, 2 μ g/ml pepstatin A, 2 μ g/ml leupeptin, 2 μ g/ml chymostatin, 2 μ g/ml antipain, 40 mM β -glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. Soluble proteins

were concentrated by precipitation with 1.5 volumes of acetone and separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by Western blot analysis with the indicated antibodies. The Ser(P)-916 and C-terminal pan-PKD antisera have been described previously and were used to detect PKD1 activity and expression levels, respectively (31). The Ser(P)-744/Ser-748 PKD antibody was purchased from Cell Signaling Technology and used at 1:1000 to detect phosphorylation of Ser-744 and Ser-748 on PKD1. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

RESULTS

PKD Ser-744 and Ser-748 Are Necessary for Optimal PKD Activation in Lymphocytes—Activation of PKD1 catalytic activity results in immediate autophosphorylation of PKD1 on a C-terminal autophosphorylation site, serine 916 (31). Accordingly, PKD1 catalytic activity *in vivo* (i.e. in an intact cell) can be monitored using antisera generated against a phosphoserine 916 peptide that selectively recognizes PKD1 molecules autophosphorylated on Ser-916. The phosphoserine 916 (Ser(P)-916) antisera has been used extensively to monitor PKD1 activity in human and murine lymphocyte cell lines and primary lymphocytes (6, 31). Western blot analysis of A20 B cell lymphoma cells with phosphoserine 916 antisera (Fig. 1A) showed that PKD1 is inactive in lymphocytes unless cells are stimulated by B cell antigen receptor ligation or after exposure to the phorbol ester phorbol 12,13-dibutyrate (PDBu), a pharmacological mimic of DAG.

PKD1 activation in fibroblasts is mediated by phosphorylation of residues Ser-744 and Ser-748 in the catalytic domain of the enzyme (14, 32). The phosphorylation of Ser-744/Ser-748 can be monitored by Western blot analysis with specific phospho-antisera. The data (Fig. 1A) show that both phorbol ester and antigen receptor activation of PKD1 in lymphocytes is accompanied by phosphorylation of Ser-744/Ser-748. Moreover, the time course of Ser-744/Ser-748 phosphorylation and PKD1 activation are coincident; the phosphorylation of Ser-744/Ser-748 and autophosphorylation of PKD1 on Ser-916 is, thus, a rapid and sustained response to antigen receptor engagement.

To determine whether phosphorylation of Ser-744/Ser-748 is necessary for PKD1 activation, the activity of a PKD1 mutant with alanine substitutions at Ser-744/Ser-748 was monitored (PKD-S744A/S748A). The PKD1 constructs used for these experiments were tagged with GFP. Western blot analysis with phosphoserine 916 antisera showed antigen receptor activation and PDBu stimulation of B cells strongly induced the catalytic activity of wild type PKD1, but this response was strikingly reduced in the PKD-S744A/S748A mutant (Fig. 1B), although it was not completely abrogated.

The Role of the PKD1 CRD in PKD1 Activation in Lymphocytes—There are two cysteine-rich motifs (C1a and C1b) within the CRD of PKD1 that are required for the enzyme to bind DAG with high affinity and control the intracellular localization of the enzyme (26, 29, 33). DAG binding to PKD is proposed to colocalize PKCs to facilitate Ser-744/Ser-748 phosphorylation, but the role of DAG binding to PKD1 for enzyme activation in lymphocytes has not been fully assessed. Previous studies have shown that PKD-P287G, a PKD1 mutant with a mutated C1b domain that has lost ~90% of its DAG/phorbol ester binding capacity, can still be activated in response to antigen receptor triggering (6, 29). However, this result does not fully resolve the role of DAG binding for PKD1 activation because PKD-P287G has residual capacity to bind DAG/phorbol esters via its low affinity C1a domain. Accordingly, we examined the activity of a second PKD1 mutant containing substitutions of prolines 155 and 287 with glycine, which disables both the C1a and C1b domains of PKD1, resulting in complete loss of DAG/phorbol ester binding. The data in Fig. 2 show Western blot analysis

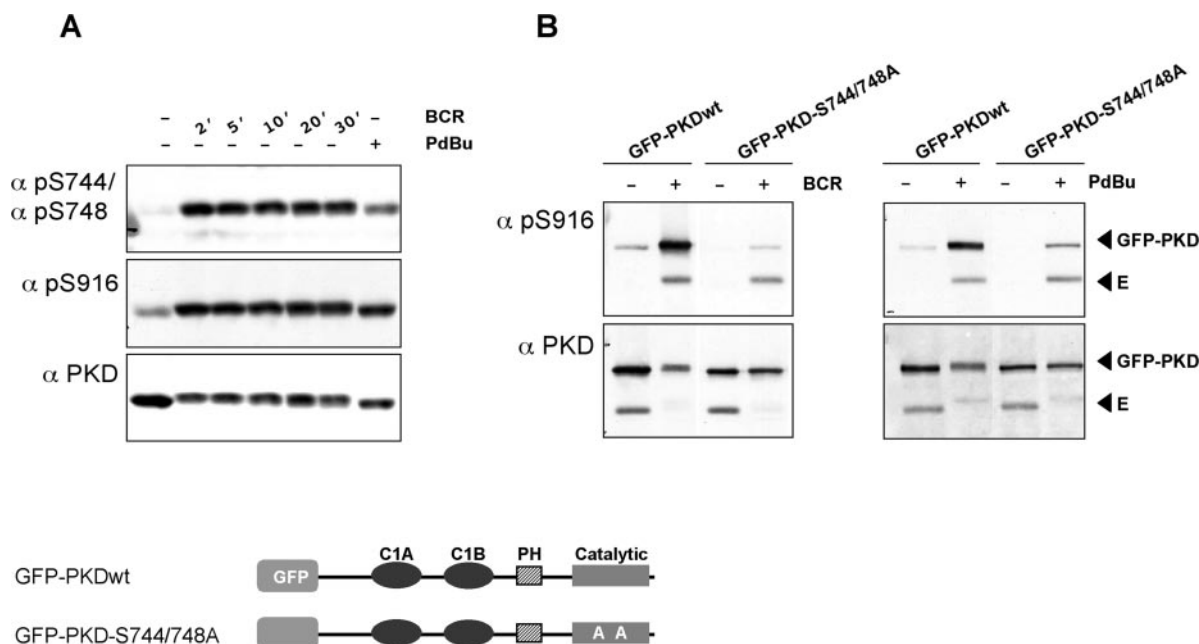


FIG. 1. Phosphorylation of Ser-744/Ser-748 is required for PKD1 activation in lymphocytes. A, A20 B lymphocytes were left unstimulated (–) or were treated with PDBu (20 ng/ml) or by cross-linking the BCR using F(ab')₂ (10 μg/ml) for the indicated time (minutes) (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α-Ser(P)-916 (α-pS916), α-Ser(P)-744/748 (α-pS744/748), and α-PKD. B, A20 B lymphocytes were transiently transfected with GFP-PKD wild type (wt) or GFP-PKD-S744A/S748A and used for experimental purposes 24 h later. Cells were left unstimulated (–) or were treated with PDBu (20 ng/ml) or by cross-linking of the BCR using F(ab')₂ (10 μg/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α-Ser(P)-916, and α-PKD. E, endogenous PKD. These results are representative of three or more independent experiments. The constructs used above are schematically depicted in this figure.

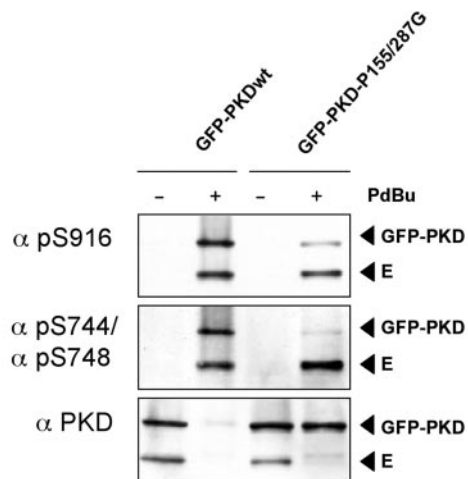


FIG. 2. The role of DAG binding in PKD1 activation in lymphocytes. DT40 cells were transiently transfected with GFP-PKD wild type (wt) and GFP-PKD-P155G/P287G and used for experimental purposes 24 h later. Cells were left unstimulated (–) or were treated with PDBu (20 ng/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α-Ser(P)-916 (α-pS916), α-Ser(P)-744/748 (α-pS744/748), and α-PKD. E, endogenous PKD. These results are representative of three or more independent experiments. The constructs used above are schematically depicted in this figure.

with Ser-916 antisera of B cells transfected with PKD-P155G/P287G and revealed that this mutant could not respond normally to phorbol ester stimulation. Thus, PKD-P155G/P287G is effectively catalytically inactive, indicating that PKD1 activa-

tion by phorbol esters in lymphocytes requires a functional DAG binding domain. The failure of PKD-P155G/P287G to respond to phorbol ester treatment correlates with the failure of this PKD1 mutant to be phosphorylated on Ser-744/Ser-748 (Fig. 2). These data are compatible with a model whereby DAG binding to PKD1 is necessary to allow the enzyme to be transphosphorylated by PKC at Ser-744/Ser-748.

Phosphorylation of PKD1 Ser-744 and Ser-748 Is Not Sufficient for PKD1 Activation in Lymphocytes—The data above indicate that phosphorylation of Ser-744/Ser-748 is important for PKD activation in lymphocytes. To explore if phosphorylation of Ser-744/Ser-748 is sufficient for PKD1 activation in lymphocytes, the activity of PKD1 molecules with acidic amino acid substitutions at Ser-744 and Ser-748 as phospho-mimics was examined (PKD-S744E/S748E). This PKD1 mutant is constitutively active in Cos7 and HeLa cells (15, 16, 23). The data in Fig. 3A compare the activity of endogenous wild type PKD1 and PKD-S744E/S748E in a stable A20 cell line expressing GFP-PKD-S744E/S748E. The data show that PKD-S744E/S748E was not constitutively active in these B cells. Rather, optimal activation of PKD-S744E/S748E was only induced in response to antigen receptor triggering or phorbol ester stimulation. The time course for activation of PKD-S744E/S748E was similar to that of endogenous PKD1; activation was both rapid and sustained for up to several hours (Fig. 3B). The activity of PKD-S744E/S748E was also examined in a T cell population, the Jurkat T leukemic cell line. Jurkat cells were transiently transfected with wild type PKD1 and PKD-S744E/S748E (Fig. 3C). There was a small increase in basal activity of the PKD-S744E/S748E, as seen in B cells, but optimal activity of this PKD1 mutant was only seen once the T cells were activated.

Deletion of the PKD1 Pleckstrin Homology (PH)-like Domain Creates a Constitutively Active Kinase in Lymphocytes—One explanation for the failure of PKD-S744E/S748E to be constitutively active in lymphocytes is that acidic substitutions as Ser-

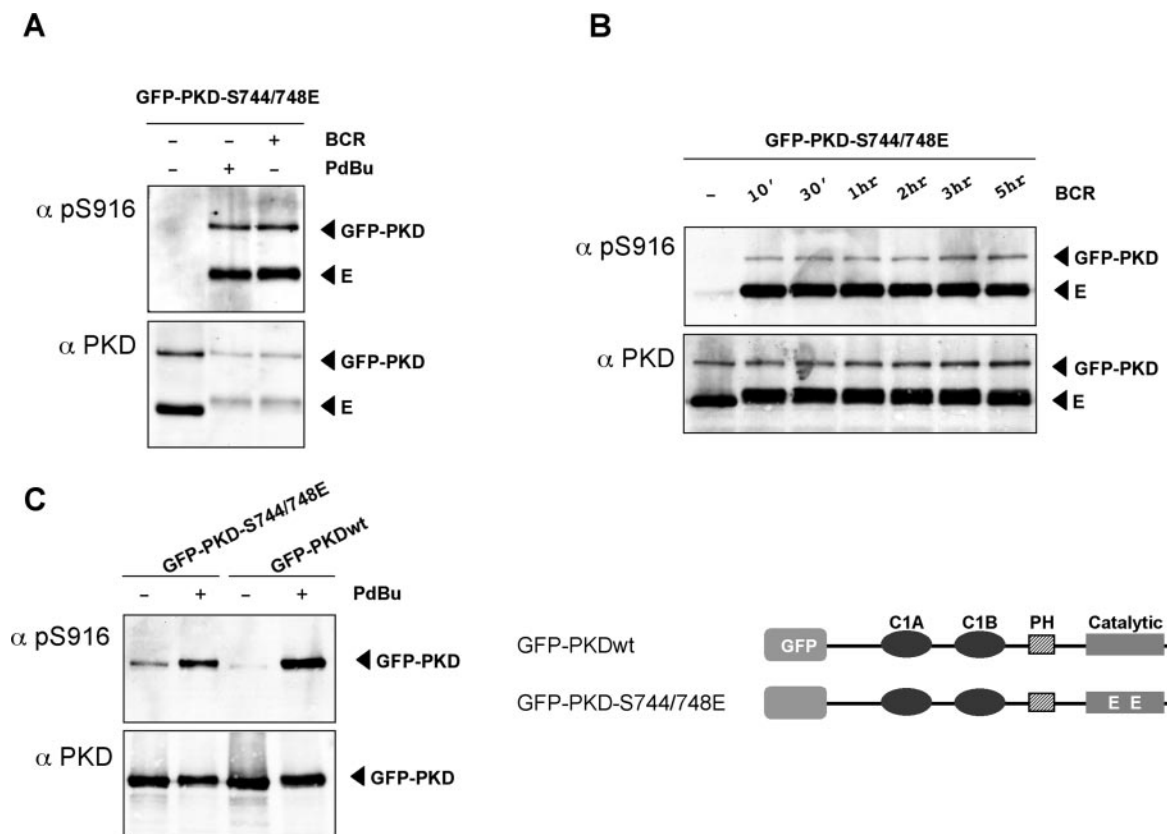


FIG. 3. Phosphorylation of Ser-744/Ser-748 is not sufficient for PKD1 activation in lymphocytes. A, a stable A20 cell line expressing the GFP-PKD-S744E/S748E construct was left unstimulated (–) or was treated with PDBu (20 ng/ml) or by cross-linking the BCR using F(ab')₂ (10 μg/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α-Ser(P)-916 (α-pS916) and α-PKD. B, a stable A20 cell line expressing GFP-PKD-S744E/S748E was left unstimulated (–) or was treated by cross-linking the BCR using F(ab')₂ (10 μg/ml) for various time points before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α-Ser(P)-916 and α-PKD. C, Jurkat cells were transiently transfected with GFP-PKD wild type (wt) or GFP-PKD-S744E/S748E and used for experimental purposes 24 h later. Cells were left unstimulated (–) or were treated with PDBu (20 ng/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α-Ser(P)-916 and α-PKD. E, endogenous PKD. These results are representative of three or more independent experiments. The constructs used above are schematically depicted in this figure.

744/Ser-748 do not act as phospho-mimics. However, PKD-S744E/S748E has been shown to be constitutively active in Cos7 cells and HeLa cells and has been used in a number of studies to probe PKD functions (16, 22, 23). The phosphorylation of Ser-744/Ser-748 is proposed to relieve N-terminal inhibition of the PKD1 catalytic domain by the PKD1 PH-like domain (14). Because acidic amino acid substitutions of Ser-744 and Ser-748 were not sufficient to activate PKD1 in lymphocytes, the impact of PKD1 PH domain deletion on PKD1 activity in lymphocytes was explored. The activities of wild type PKD1, PKD-S744E/S748E, and PKDΔPH, a PKD1 construct with a deletion of the PH domain (amino acids 429 to 557), were compared (Fig. 4). These constructs were all fused to the C terminus of GFP so that they could be readily distinguished from endogenous PKD1. The different PKD1 mutant constructs were transiently transfected (separately) into A20 cells, and their activity was analyzed by monitoring autophosphorylation of Ser-916. The data (Fig. 4) show that PKDΔPH exhibited substantial basal activity in unstimulated cells, whereas wild type PKD1 (both endogenous and GFP-tagged) and PKD-S744E/S748E were not. Activation of lymphocytes with phorbol ester robustly stimulated the activity of wild type PKD1 and PKD-S744E/S748E. In contrast, phorbol ester stimulation did not further enhance the activity of PKDΔPH, indicating that this mutant is maximally active when expressed in lymphocytes.

The Role of PLC and PKC in Antigen Receptor Regulation of Wild Type PKD1 Versus PKD-S744E/S748E—Antigen receptor regulation of PKD1 is mediated by a PLC/DAG/PKC pathway

(8). Ser-744 and Ser-748 in PKD1 are direct substrates for PKCs (14), and it has been proposed that the PLC/PKC requirement for antigen receptor activation of PKD1 is wholly explained by the requirement of these molecules for antigen receptor-induced phosphorylation of PKD1 at Ser-744/Ser-748. To test this hypothesis we examined Ser-744/Ser-748 phosphorylation of PKD1 in a DT40 B cell line where the antigen receptor-coupled PLC, PLCγ2, was ablated by homologous recombination, and the BCR is unable to induce hydrolysis of phosphatidylinositol 4,5-diphosphate and, hence, cannot generate inositol polyphosphates or DAG (30). DT40 and DT40 PLCγ2^{-/-} cells were stimulated via cross-linking the BCR or by treatment with PDBu. The data (Fig. 5A) show that PKD1 is not phosphorylated on Ser-744/Ser-748 in quiescent DT40 cells. In wild type DT40 cells both BCR and PDBu stimulation results in the phosphorylation of PKD1 on Ser-744/Ser-748. In PLCγ2^{-/-} DT40s, BCR stimulation was unable to induce DAG production (the physiological stimulus for PKC) and was also unable to induce phosphorylation of PKD1 at Ser-744/Ser-748 (Fig. 5A). However, PDBu (a pharmacological mimic of DAG that can bypass the loss of PLCγ to activate PKC directly) induced a robust PKD1 Ser-744/Ser-748 phosphorylation in PLCγ2^{-/-} DT40 cells.

To probe the involvement of PKCs in Ser-744/Ser-748 phosphorylation of PKD1 in lymphocytes, the effects of the PKC inhibitor GF109203X on either PDBu- or BCR-stimulated DT40 cells was examined. The catalytic domains of PKCs and PKD are so divergent that PKC inhibitors such as GF109203X

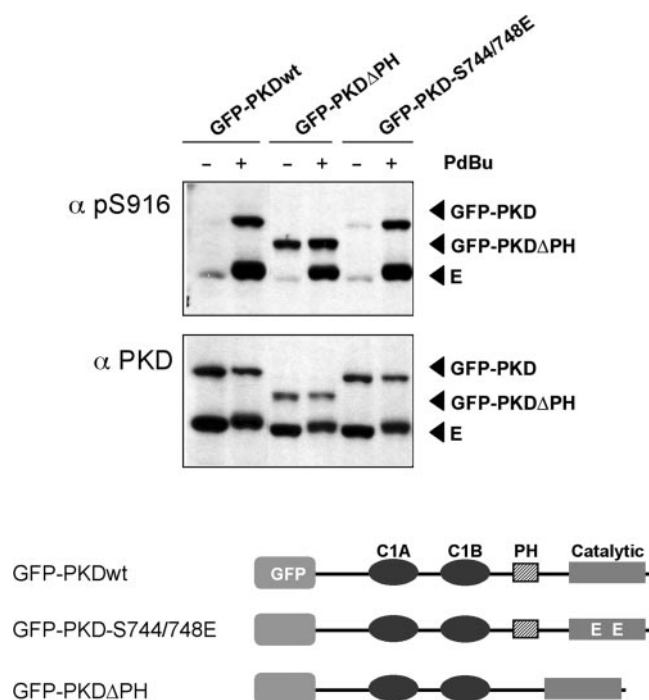


FIG. 4. **Comparisons of activity across various PKD1 mutants.** A20 cells were transiently transfected with GFP-PKD wild type (*wt*), GFP-PKD Δ PH, or GFP-PKD-S744E/S748E and used for experimental purposes 24 h later. Cells were left unstimulated (–) or were treated with PDBu (20 ng/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α -Ser(P)-916 (α -pS916) and α -PKD. E, endogenous PKD. These results are representative of three or more independent experiments. The constructs used above are schematically depicted in this figure.

have no direct action on PKD (34). However, the data (Fig. 5A) showed that phosphorylation of PKD1 on Ser-744/Ser-748 in BCR- and PDBu-treated wild type DT40 cells is prevented by the addition of the PKC inhibitor GF109203X. In DT40 cells lacking PLC γ 2 only PDBu activation resulted in phosphorylation of Ser-744/Ser-748, and this response was also prevented by GF109203X. Antigen receptor-induced phosphorylation of PKD1 on Ser-744/Ser-748 was, thus, dependent on both PLC and PKC. Phorbol ester-induced phosphorylation of PKD1 was only dependent on PKC.

The results above indicate that PKD1 activation in lymphocytes conforms in part to the models derived from fibroblast experiments, namely, that PLC and PKC regulate PKD1 via phosphorylation of Ser-744 and Ser-748. However, there is the question of what signaling pathways are used by antigen receptors to regulate the activity of the PKD-S744E/S748E mutant in which the key PKC substrate serines are substituted with glutamic acid as a phospho-mimic. To explore this issue wild type or PLC γ 2 null DT40 cells were transfected with the PKD-S744E/S748E mutant and subsequently stimulated with either PDBu or BCR cross-linking. PKD1 activity was monitored by Western blot analysis with the Ser(P)-916 antisera. The data show that in wild type DT40 cells the PKD-S744E/S748E mutant has low basal activity but can be strongly activated by either BCR ligation or PDBu treatment. In PLC γ 2^{–/–} DT40 cells PKD-S744E/S748E could not be activated by BCR ligation, although it remained sensitive to PDBu (Fig. 5B).

PDBu could activate PKD-S744E/S748E by directly binding to the enzyme itself, or alternatively, PDBu could induce PKC mediated phosphorylations of PKD1 at sites additional to Ser-744/748. The PKC requirement for PDBu activation of PKD-S744E/S748E was, therefore, investigated by examining the effects of the PKC inhibitor GF109203X on PDBu stimulation

of the PKD-S744E/S748E mutant. The data (Fig. 5C) showed that PDBu-mediated activation of PKD-S744E/S748E is insensitive to the addition of GF109203X. This is in marked contrast to the effects of the PKC inhibitor on the endogenous enzyme; activation of endogenous wild type PKD1 was abrogated when cells were treated with GF109203X (Fig. 5C). The effect of GF109203X on antigen receptor activation of PKD-S744E/S748E was also examined. The data (Fig. 5D) confirmed that antigen receptor activation of endogenous PKD1 was inhibited when cells were pretreated with GF109203X but that the PKD-S744E/S748E mutant remained maximally active under these conditions. PKD-S744E/S748E is, thus, no longer dependent on PKC for activation.

DISCUSSION

The present data reveal that PKD1 activation in lymphocytes requires PKC-mediated phosphorylation of Ser-744 and Ser-748. Hence, inhibition of PKC or mutation of these two serines to alanine inhibits antigen receptor activation of PKD1 (6, 7). The requirement for PKC phosphorylation of Ser-744 and Ser-748 in PKD1, thus, seems to be essential for PKD1 activation in a variety of cell lineages and for diverse stimuli. However, the present study has found a key difference between PKD1 regulation in lymphocytes and some other cell types. Notably, mutation of Ser-744 and Ser-748 to glutamic acid as phospho-mimics is sufficient for PKD1 activation in HeLa and Cos7 cells (15, 16, 35). Accordingly, PKD-S744E/S748E is used increasingly as a constitutively active mutant to probe the cellular function of PKD (16, 22, 23). The data herein show PKD-S744E/S748E was not constitutively active in lymphocytes but remained dependent on antigen receptor triggering or phorbol ester stimulation for optimal activation. The present results, thus, make two key points; first, PKD1 activation mechanisms differ in different cells, and second, PKD-S744E/S748E should be used cautiously as a research tool as it is not constitutively active in all cells.

It has been proposed that the phosphorylation of activation loop serines 744 and 748 in the PKD1 catalytic domain relieves PKD1 PH domain-mediated autoinhibition and stabilizes PKD1 in an active conformation (14). The PKD1 PH domain has an autoinhibitory action on PKD1 catalytic function in lymphocytes as its deletion creates a constitutively active enzyme. The low basal activity of the PKD-S744E/S748E mutant compared with the high activity of PKD Δ PH in lymphocytes is consistent with a model whereby the catalytic domain of PKD-S744E/S748E is still subject to N-terminal inhibition in quiescent lymphocytes and that antigen receptor or phorbol ester stimulation activates PKD-S744E/S748E by relieving this N-terminal inhibition. Experiments in PLC γ 2 null lymphocytes show that antigen receptor-induced activation of PKD-S744E/S748E and the wild type enzyme were both dependent on PLC stimulation. Hence, PLC can regulate PKD1 independent of its role in regulating PKC-mediated transphosphorylation of Ser-744/Ser-748. Strikingly, antigen receptor or phorbol ester stimulation of PKD-S744E/S748E is not prevented by pretreatment of cells with PKC inhibitors, in marked contrast to the behavior of the wild type enzyme. Hence, mutation of PKD1 Ser-744 and Ser-748 to glutamic residues is a phospho-mimic substitute for PKC but not antigen receptor activation. PKC-mediated phosphorylation of Ser-744/Ser-748 is, thus, not sufficient to explain antigen receptor activation of PKD1, and an additional antigen receptor/PLC generated signal is required that is independent of PKC.

The PLC-dependent/PKC autonomous step for PKD1 activation is mediated by DAG since phorbol esters, pharmacological mimics of DAG, can bypass the loss of PLC and stimulate PKD-S744E/S748E activation in PLC γ null lymphocytes. Previously, the role of DAG/phorbol esters in PKD1 activation was

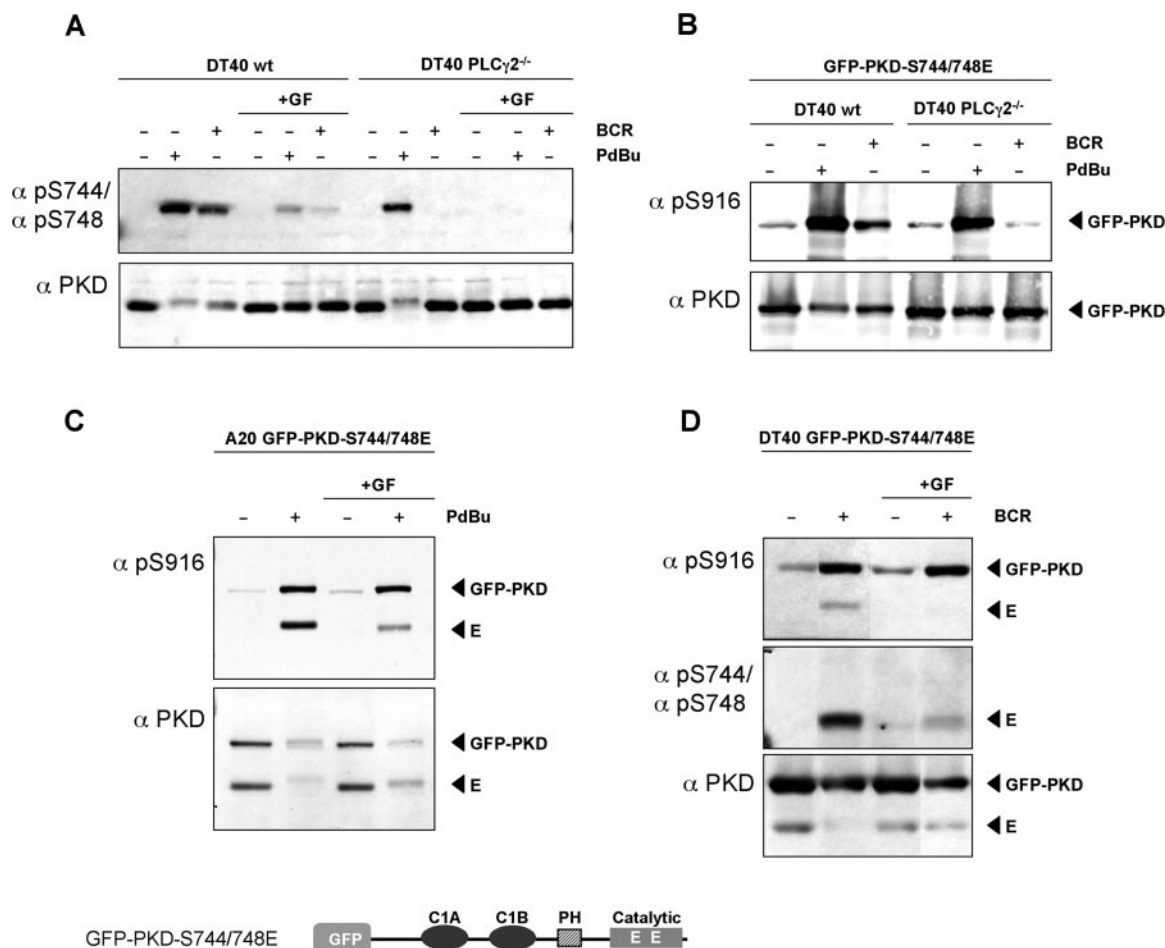


FIG. 5. Role of PLC γ 2 and PKC in the activation of PKD1 wild type and PKD-S744E/S748E. A, DT40 wild type (*wt*) and DT40 PLC γ 2^{-/-} cells were pretreated with GF109203X (*GF*) (5 μ M) for 20 min, then left unstimulated (-) or were treated with PDBu (20 ng/ml) or cross-linking of the BCR using IgM monoclonal antibody M4 (20 μ g/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α -Ser(P)-744/748 (α -pS744/ α -pS748) and α -PKD. B, DT40 wild type and DT40 PLC γ 2^{-/-} cells were transiently transfected with GFP-PKD-S744E/S748E and used for experimental purposes 24 h later. Cells were left unstimulated (-) or were treated with PDBu (20 ng/ml) or by cross-linking the BCR using IgM monoclonal antibody M4 (20 μ g/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α -Ser(P)-916 (α -pS916) and α -PKD. C, A20 cells were transiently transfected with GFP-PKD-S744E/S748E and used for experimental purposes 24 h later. Cells were pretreated with GF109203X (5 μ M) for 20 min, then left unstimulated (-) or were treated with PDBu (20 ng/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α -Ser(P)-916 and α -PKD. D, DT40 cells were transiently transfected with GFP-PKD-S744E/S748E and used for experimental purposes 24 h later. Cells were pretreated with GF109203X (5 μ M) for 20 min, then left unstimulated (-) or were treated by cross-linking the BCR using IgM monoclonal antibody M4 (20 μ g/ml) for 10 min (+) before lysis and acetone precipitation. Proteins were separated by SDS-PAGE and Western-blotted using α -Ser(P)-916 and α -PKD. E, endogenous PKD. Representative of three or more independent experiments. The constructs used above are schematically depicted in this figure.

attributed to a DAG requirement for PKC activation and colocalization with PKD1 (17). A simple hypothesis to explain PKC-independent but DAG-mediated PKD1 activation is that direct binding of DAG to the CRD of PKD-S744E/S748E is required to release the PKD1 catalytic domain from N-terminal inhibition. The caveat is that the PKD1 CRD domain plays a key role in determining the subcellular localization of the enzyme, and it is possible that DAG binding to the CRD of PKD-S744E/S748E correctly localizes the enzyme for additional regulation by some other stimulus. In this respect, PKD1 activation in lymphocytes does not appear to require plasma membrane translocation of the enzyme since mutation of the C1b domain alone, which prevents relocation of PKD1 to the plasma membrane, does not prevent antigen receptor activation of the enzyme (6). As well, PKD1 activation precedes plasma membrane translocation in lymphocytes and appears to occur in the cell interior (7).

A requirement for PLC activation and DAG production independent of the DAG requirement for PKC-mediated phosphorylation of PKD1 has not been previously recognized in HeLa

cells and Cos7 cells (15, 16) because substitutions of acidic residues for Ser-744/Ser-748 is sufficient to activate PKD1 in these cells. This would suggest that there is sufficient endogenous DAG to activate PKD-S744E/S748E in some cell populations, whereas in lymphocytes endogenous DAG levels are limiting in the absence of antigen receptor engagement. Many factors determine levels of endogenous DAG including the relative expression and activity of DAG kinases. In this instance DAG kinases are known to be important regulatory enzymes in lymphocytes (36, 37) and may contribute to keeping DAG levels low in quiescent cells. Another factor to consider is that the ability of endogenous DAG to saturate PKD1 will be influenced by the intracellular concentration of PKD1. It could, thus, be relevant that PKD1 is extremely abundant in lymphocytes and is expressed at 10–100-fold higher levels than in most fibroblasts and epithelial cells.² Hence, even if endogenous DAG levels are equal, DAG will be rate-limiting in a cell with very

² C. D. Wood, unpublished data.

high levels of PKD1. A further complication is that cells can express multiple DAG-binding proteins including the different DAG kinase isoforms, PKCs, and the Ras guanine nucleotide exchange proteins Ras guanyl nucleotide-releasing proteins (38). DAG availability to PKD1 would inevitably be influenced by the presence of these competing DAG-binding proteins, and DAG might be rate-limiting for PKD1 activation in cells where the ratio of PKD1 to other DAG-binding proteins is low. It could also be significant that PKD1 can have quite different patterns of intracellular location in different cell lineages (24–26), and different intracellular membranes may contain different molecular species of DAG that differ in their affinity for PKD1. Different positioning of PKDs inside cells would inevitably influence the basal levels of DAG to which the enzyme is exposed. For example, HeLa cells are one model system in which PKD-S744E/S748E mutant is constitutively active (16). These cells express very low levels of PKD1 relative to lymphocytes and also exhibit spontaneous trans-Golgi localization of PKD1 that is mediated by the CRD or DAG/phorbol ester binding region of the enzyme (33). If there is sufficient DAG in trans-Golgi membranes of HeLa cells to recruit PKD1, this could also be sufficient to basally activate the PKD-S744E/S748E mutant. By contrast, in quiescent lymphocytes PKD1 is cytosolic and not discernibly in contact with endogenous membranes, Golgi or otherwise, before antigen receptor triggering (7). This is an indication that PKD1 does not spontaneously bind DAG in quiescent lymphocytes either due to very low basal levels of DAG in lymphocyte membranes or because there are other competing DAG-binding proteins already *in situ* that prevent DAG/PKD1 interactions.

In summary, the present data describe PKD1 regulatory mechanisms in antigen receptor-activated lymphocytes and reveal that PKD1 is not regulated by DAG and PKC in a linear pathway; rather, PKD1 activation has more stringent requirements for integration of two DAG signals, one mediated by PKCs and the other independent of this family of serine kinases. This second pathway can either be intrinsic or be regulated by extracellular stimuli and probably reflects a requirement for DAG binding to the PKD1 CRD to relieve N-terminal inhibition of the PKD1 catalytic domain.

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Dual Phospholipase C/Diacylglycerol Requirement for Protein Kinase D1 Activation in Lymphocytes

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