The protein kinase D1 COOH terminus: marker or regulator of enzyme activity?

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Qiu W, Zhang F, Steinberg SF. The protein kinase D1 COOH terminus: marker or regulator of enzyme activity? Am J Physiol Cell Physiol 307: C606–C610, 2014. First published July 30, 2014; doi:10.1152/ajpcell.00155.2014.—Protein kinase D1 (PKD1) is a Ser/Thr kinase implicated in a wide variety of cellular responses. PKD1 activation is generally attributed to a PKC-dependent pathway that leads to phosphorylation of the activation loop at Ser744/Ser748. This modification increases catalytic activity, including that toward an autoprophosphorylation site (Ser916) in a postsynaptic density-95/disks large/zonula occludens-1 (PDZ)-binding motif at the extreme COOH terminus. However, there is growing evidence that PKD1 activation can also result from a PKC-independent autocatalytic reaction at Ser744/Ser748 and that certain stimuli increase PKD1 phosphorylation at Ser744/Ser748 without an increase in autoprophosphorylation at Ser916. This study exposes a mechanism that results in a discrepancy between PKD1 COOH-terminal autoprophosphorylative activity and activity toward other substrates. We show that PKD1 constructs harboring COOH-terminal epitope tags display high levels of in vitro activation loop autoprophosphorylation activity and activity toward syntide-2 (a peptide substrate), but no Ser916 autocatalytic activity. Cell-based studies show that the COOH-terminal tag, adjacent to PKD1’s PDZ1-binding motif, does not grossly influence PKD1 partitioning between soluble and particulate fractions in resting cells or PKD1 translocation to the particulate fraction following treatment with PMA. However, a COOH-terminal tag that confers a high level of activation loop autoprophosphorylation decreases the PKC requirement for agonist-dependent PKD1 activation in cells. The recognition that COOH-terminal tags alter PKD1’s pharmacological profile is important from a technical standpoint. The altered dynamics and activation mechanisms for COOH-terminal-tagged PKD1 enzymes also could model the signaling properties of localized pools of enzyme anchored through the COOH terminus to PDZ domain-containing scaffolding proteins.

protein kinase D; postsynaptic density-95/disks large/zonula occludens-1-binding motif; phosphorylation

MATERIALS AND METHODS

Materials. The anti-PKD-Ser(P)744/Ser(P)748 phosphorylation state-specific antibody that preferentially recognizes phosphorylation at Ser744 was obtained from Cell Signaling Technology and the anti-PKD-Ser(P)742 (numbering based on human sequence, corresponding to rodent PKD1-Ser748) from Abcam. Other antibodies were obtained from Cell Signaling Technologies. Syntide-2 was obtained from Sigma.

PKD1 mutants. Plasmids that drive expression of HA-tagged wild type (WT)-PKD1 and PKD1-S916A generated by the Toker laboratory were obtained from Addgene. A plasmid that drives expression of full-length PKD1 with GFP fused to the COOH terminus was generously provided by Drs. David E. Clapham and Elena Oancea (6). WT-PKD1 and PKD1-S916A constructs with a FLAG tag fused to the COOH terminus were generated by PCR and validated by sequencing.
PKD1 expression plasmids were introduced into human embryonic kidney (HEK 293) cells by Effectene transfection reagent (Qiagen) according to the instruction manual. Cells were grown for 24 h and lysed in RIPA buffer containing 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml benzamidine, 0.5 mM PMSE, 5 μM pepstatin A, and 0.1 μM calcium. Cell extracts were used for immunoblotting experiments or subjected to immunoprecipitation with anti-HA (Roche Applied Science), mouse monoclonal anti-GFP 3E6 (Invitrogen), or anti-FLAG (Sigma) for subsequent in vitro kinase assays.

In vitro kinase assays. In vitro kinase assays were performed with PKD1 immunoprecipitated from 150 mg of starting cell extract. Incubations were performed in 110 μl of a reaction buffer containing 30 mM Tris·Cl, pH 7.5, 5.45 mM MgCl2, 0.65 mM EDTA, 0.65 mM EGTA, 0.1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 0.1 μM calyculin, 0.55 μM protein kinase inhibitor peptide, 217 mM NaCl, 3.6% glycerol, and [γ-32P]ATP (10 μCi, 66 μM). The reactions continued for 30 min (or as indicated) at 30°C in buffers containing vehicle or 89 μg/ml phorbol ester and 10 μg/ml aprotinin. The reactions were terminated by addition of 30 mM phosphoric acid, washed (3 times for 5 min), dried, and counted for radioactivity.

RESULTS

Current concepts regarding the molecular determinants that control PKD1 activity are based largely on studies that interrogate the biochemical properties and cellular actions of epitope-tagged PKD1 mutants. The general approach has been to use GFP (or red fluorescent protein)-tagged enzymes to visualize dynamic changes in the subcellular distribution of the enzyme in agonist-stimulated cells or HA-tagged constructs to examine the in vitro biochemical properties of the enzyme; the HA tag provides a particularly convenient handle to purify the enzyme and avoid contamination with endogenous kinases. The general consensus of these studies is that epitope tags do not detectably alter the phorbol ester-binding affinity, activation loop phosphorylation, or catalytic activity of the enzyme (5, 8, 18).

In this context, Fig. 1 shows a heretofore unrecognized difference in the phosphorylation profiles and catalytic activities of NH2-terminal (HA)-tagged vs. COOH-terminal (FLAG or GFP)-tagged PKD1 enzymes. The NH2-terminal HA-tagged enzyme is recovered from resting HEK 293 cells with little-to-no activation loop or Ser916 phosphorylation; autophosphorylation at both activation loop sites and the COOH terminus increases upon treatment with PMA (Fig. 1). HA-PKD1-Ser916 autophosphorylation is a very rapid reaction that is completed within the first 5 min of incubation (Fig. 1B).

Overall HA-PKD1 autophosphorylation (quantified as 32P incorporation by PhosphoImager) and HA-PKD1 autophosphorylation at Ser744/Ser748 (measured by immunoblot analysis) is delayed in comparison; these measures of HA-PKD1 autophosphorylation are detected at low levels at 20 min and reach a maximal level only following 45–60 min of incubation. HA-PKD1 activity toward syntide-2 also is detected only following a 20- to 30-min lag, consistent with the notion that substrate phosphorylation also requires prior HA-PKD1 autophosphorylation at Ser744/Ser748.

The phosphorylation profiles of PKD1 enzymes harboring COOH-terminal epitope tags (FLAG or GFP) are quite different. The COOH-tagged PKD1-FLAG or PKD1-GFP enzymes are recovered from cells with some constitutive phosphorylation at the activation loop (particularly at Ser744), PKD1-FLAG or PKD1-GFP enzymes display high levels of PMA-dependent autophosphorylation, detected as a marked increase in 32P incorporation and high levels of autophosphorylation at both activation loop sites (Ser744 and Ser748). It should be noted that 32P incorporation into the various PKD1 constructs is an integrated measure of autophosphorylation at Ser744/Ser748 and possibly other autophosphorylation sites elsewhere in the enzyme [including Ser205/Ser208, Ser210/Ser223, and Ser249 (17)]. Autophosphorylation at these other sites contributes to 32P incorporation but is not detected in the Western blot studies. Kinetic studies show that the COOH-terminal tag accelerates the kinetics and amplitude of PKD1 autophosphorylation and PKD1-dependent phosphorylation of syntide-2 relative to the NH2-terminal HA-tagged enzyme. PKD1-FLAG also displayed increased activity toward the endogenous protein substrate cAMP response element-binding protein (3.1 ± 0.2-fold vs. HA-PKD1, n = 5, P < 0.05), which is a physiologically relevant substrate of PKD (7, 12). However, COOH-tagged PKD1-FLAG or PKD1-GFP enzymes do not autophosphorylate at Ser916, even with prolonged (up to 60 min) incubation intervals.

We previously demonstrated that a S916A substitution in the NH2-terminal HA-tagged enzyme disrupts Ser748 autocatalytic activity (10). This phenotype (which is reproduced in Fig. 2A) is in marked contrast to the phenotype of the COOH-terminal-tagged enzyme, where a Ser916 autophosphorylation defect does not prevent activation loop autophosphorylation at Ser748. In theory, this discrepancy might be due to an artifact if a COOH-terminal tag interferes with immunodetection of adjacent sequence at the COOH terminus of the enzyme. This trivial explanation is effectively countered by the observation that the PKD1-FLAG enzyme is readily detected by an antibody that recognizes sequence at the COOH terminus of PKD1 (Figs. 1 and 2). A previous study showed that a COOH-terminal-directed PKD1 antibody also recognizes PKD1-GFP (1). The trivial explanation also is refuted by additional experiments showing that a S916A substitution in the PKD1-FLAG backbone does not abrogate Ser748 autophosphorylation (Fig. 2B). Collectively, these results argue that a COOH-terminal epitope results in a bone fide Ser916 autophosphorylation defect that does not disrupt Ser748 autocatalytic activity.

We performed cell fractionation studies to determine whether a COOH-terminal tag, adjacent to the PDZ1-binding motif, influences PKD1 translocation to particulate membranes. Figure 3A shows that HA-PKD1 and PKD1-FLAG enzymes partition similarly between soluble and particulate
fractions in resting cells and that both enzymes are detected exclusively in the particulate fraction following treatment with PMA. These subcellular localization patterns for HA-PKD1 and PKD1-FLAG enzymes recapitulate the subcellular partitioning patterns described for native PKD1, indicating that epitope tags do not grossly alter subcellular localization patterns of the enzyme.

Figure 3B shows that PMA treatment leads to increased Ser744/Ser748 phosphorylation on HA-PKD1 and PKD1-FLAG enzymes, but the PMA-dependent increase in Ser916 phosphor-

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Fig. 1. NH2- and COOH-terminal tags exert distinct effects on in vitro PKD1 autophosphorylation and syntide-2 kinase activity. PKD1 constructs harboring NH2 (HA) or COOH (FLAG or GFP) terminal tags were immunoprecipitated from human embryonic kidney (HEK 293) cells and subjected to in vitro kinase assays for 20 min (A) or 0–60 min (B) without or with phosphatidylserine (PS)/PMA; assays with PS/PMA were performed in duplicate in A. PKD1 autocatalytic activity was tracked by autoradiography (quantified as 32P incorporation, with results in B reported as means ± SE, n = 4) or by immunoblot analysis with phosphorylation state-specific antibodies that track phosphorylation at individual sites in the activation loop or at Ser916 (the autophosphorylation site at the extreme COOH terminus). Identity and location of epitope tags relative to the modular domain structure of PKD1 are illustrated in A (bottom). C1, lipid-binding C1 domain; PH, autoinhibitory pleckstrin homology; KD, kinase domain.

Fig. 2. PKD1-FLAG displays a Ser916 autophosphorylation defect, but no associated defect in activation loop autophosphorylation at Ser748. HA-PKD1, HA-PKD1-S916A, PKD1-FLAG, and PKD1-S916A-FLAG were immunoprecipitated from HEK 293 cells and subjected to in vitro kinase assays (IVKAs) for 20 min without or with PS/PMA. PKD1 protein and phosphorylation were tracked as described in Fig 1. Dashed lines in A denote where data from different regions of a single gel were merged for purposes of presentation.
PKD1-Ser744/Ser748 phosphorylation; this response is mediated by bin (an agonist for the endogenous G protein-dependent mechanism for PKD1 activation. Figure 3 shows that thrombin-induced PKD1-Ser916 autophosphorylation defect identified in the in vitro studies is retained in the in vivo context.

Finally, we identified an effect of the COOH tag on the mechanism for PKD1 activation. Figure 3C shows that thrombin (an agonist for the endogenous G protein-dependent pro tease-activated receptor) induces a robust increase in PKD1-Ser744/Ser748 phosphorylation; this response is mediated by a PKC-dependent pathway that is inhibited by GF109203X. In contrast, the thrombin-dependent increase in Ser744/Ser748 phosphorylation on the PKD1-FLAG enzyme (which already displays some constitutive Ser744/Ser748 phosphorylation in resting cells) is via a PKC-independent mechanism that is not inhibited by GF109203X. Since the GF109203X pretreatment protocol used in our studies effectively inhibits PKC activity (11), these results argue that a COOH-terminal epitope (which confers a high level of activation loop autophosphorylation) impacts on the PKC requirement for agonist-dependent PKD1 activation in cells.

DISCUSSION

In the 20 years since PKD1 was first identified as a lipid-activated signaling kinase, it has been implicated in a wide range of fundamental biological processes that regulate cell proliferation, differentiation, apoptosis, immune regulation, cardiac contraction, cardiac hypertrophy, angiogenesis, and cancer (9, 14). The emergence of PKD1 as a mediator of various physiological and pathological cellular responses has driven efforts to understand the mechanisms underlying PKD1’s cellular actions and, particularly, the regulatory phosphorylations that control PKD1 activity. Our current understanding of the regulatory phosphorylation that accompanies (or is required for) PKD1 activation is based largely on studies with heterologously overexpressed WT or mutant PKD1 enzymes harboring epitope tags. Most studies have used PKD1 vectors containing an epitope tag (HA, GFP, or cMyc) at the NH2 terminus. There is considerable evidence that PKD1 tolerates epitope tags at its NH2 terminus; NH2-terminal epitope tags do not grossly influence PKD1 translocation or agonist-dependent changes in PKD1 phosphorylation (13, 17). The experience with PKD1 constructs harboring a COOH-terminal tag is considerably more limited. Previous studies used PKD1-GFP primarily to track PKD1 localization and signaling responses in specific subcellular compartments (2, 6).

A comprehensive analysis of the possible confounding effects of the COOH-terminal epitope tag has not been published. This study identifies two major effects of a COOH-terminal epitope tag: we show that 1) PKD1-GFP and PKD1-FLAG enzymes exhibit a PKD1-Ser916 autophosphorylation defect, exposing a condition that results in an agonist-dependent increase in PKD1 catalytic activity that is not accompanied by an increase in PKD1-Ser916 phosphorylation and 2) the COOH-terminal-tagged enzymes display enhanced activation loop autophosphorylation and increased syntide-2 kinase activity (compared with HA-PKD1). The similar results for COOH-terminal GFP- and FLAG-tagged enzymes indicate that it is the location, rather than the identity of the tag, that is the factor. The coordinate changes in activation loop and Ser916 phosphorylation are presumed to be the direct consequence of the tag at the COOH terminus. However, more elaborate regulatory controls that arise through indirect mechanisms (e.g., a conformational change due to autophosphorylation at one site leading to a change in autocatalytic activity toward another site in the enzyme) are possible and might be considered in future studies.

The identification of an altered pharmacological profile for a COOH-terminal-tagged PKD1 enzyme is important from a technical standpoint. A COOH-terminal epitope tag is predicted to induce a similar change in the pharmacological profile of PKD2 (which is structurally similar to PKD1). Effects on PKD3 are less predictable, since PKD3 lacks the conserved COOH-terminal autophosphorylation site and PDZ-binding motif; this structural difference between the extreme COOH-terminal regions of PKD2 and PKD3 has been implicated in their distinct subcellular localization patterns (7). The relevance of these findings to the control of other enzyme families is uncertain. While structural determinants at the COOH terminus of AGC protein kinases (e.g., PKA, PKC, and AKT) function as essential components (or key allosteric regulators) of the kinase machinery (3) and there is evidence that the extreme COOH terminus is critical for the catalytic competence and biological functions of several PKC family enzymes (15, 19–21), studies to date indicate that COOH-terminal tags do not grossly alter the regulatory controls for PKC6. Some COOH-terminal tags are well tolerated.

Finally, it is tempting to speculate that PKD1 constructs harboring a COOH-terminal epitope might model a pool of enzyme that is anchored via its COOH-terminal PDZ domain-interacting motif to a PDZ domain-containing scaffolding protein. Kunzel et al. (4) identified a phorbol-12,13-dibutyrate-induced interaction between PKD1’s PDZ-binding motif and the PDZ domain-containing scaffold Na+ /H+ exchanger reg-

Fig. 3. A: COOH-terminal FLAG tag influences the activation profile, but not the subcellular localization, of PKD1. HEK 293 cells that heterologously overexpress HA-PKD1 or PKD1-FLAG were treated with vehicle or PMA (300 nM), thrombin [Thr (1 U/ml)], or thrombin + GF109203X [GFX (10 μM)]. Soluble (S) and particulate (P) fractions (A) or whole cell lysates (B and C) were subjected to immunoblot analysis for PKD1 protein and PKD1 phosphorylation.
ulteratory factor 1 (NHERF-1). By using a series of genetically encoded PKD activity reporters, these investigators showed that the dynamics of agonist-dependent PKD1 activation at the membrane-localized NHERF-1 scaffold is markedly accelerated compared with PKD activation in other cellular compartments (4). Such accelerated activation kinetics for the PDZ-1-interacting domain-anchored enzyme were attributed to PKD1 colocalization with its activator PKCδ at the NHERF-1 scaffold. While the notion that NHERF-1 functions as a platform to colocalize PKD1 with PKCδ is quite reasonable, previous studies did not provide direct evidence that the accelerated activation kinetics for NHERF-1-associated PKD require PKC activity. In this regard, our studies suggest an alternative mechanism, namely, that PKD1’s COOH terminus imposes a structural constraint that functions to limit PKD1’s intrinsic catalytic activity and that a protein-protein interaction involving the COOH terminus (either the introduction of a COOH-terminal epitope or a docking interaction involving the COOH-terminal PDZ-binding domain) results in a conformational change that relieves this autoinhibitory constraint and increases PKD1’s intrinsic catalytic activity. A catalytically active PKD1 that autophosphorylates at the activation loop would have a decreased requirement for PKC.

Collectively, these studies provide evidence that molecular determinants at the extreme COOH terminus of PKD1 function as regulators, and not just passive markers, of PKD1 activity, suggesting that structural determinants at the COOH terminus can be targeted for therapeutic advantage.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
W.Q. and F.Z. performed the experiments; W.Q., F.Z., and S.F.S. analyzed the data; W.Q., F.Z., and S.F.S. prepared the figures; F.Z. and S.F.S. interpreted the results of the experiments; S.F.S. conception and design of the research; S.F.S. drafted the manuscript; S.F.S. edited and revised the manuscript; S.F.S. approved the final version of the manuscript.

REFERENCES