A Novel Tyrosine Phosphorylation Site in Protein Kinase D Contributes to Oxidative Stress-mediated Activation

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Protein kinase D1 (PKD1) is a mediator of oxidative stress signaling where it regulates cellular detoxification and survival. Critical for the regulation of PKD1 activity in response to oxidative stress are Src- and Abl-mediated tyrosine phosphorylations that eventually lead to protein kinase Cδ (PKCδ)-mediated activation of PKD1. Here we identify Tyr95 in PKD1 as a previously undescribed phosphorylation site that is regulated by oxidative stress. Our data suggest that PKD1 phosphorylation at Tyr95 generates a binding motif for PKCδ, and that oxidative stress-mediated PKCδ/PKD interaction results in PKD1 activation loop phosphorylation and activation. We further analyzed all PKD isoforms for this mechanism and show that PKD enzymes PKD1 and PKD2 are targets for PKCδ in response to oxidative stress, and that PKD3 is not a target because it lacks the relevant tyrosine residue that generates a PKCδ interaction motif.

Protein kinase D (PKD)2 is a serine/threonine kinase that belongs to the family of calcium/calmodulin-dependent kinases (1, 2). The PKD kinase family consists of three members, PKD1/PKCμ, PKD2, and PKD3/PKCγ, which have some overlapping but also distinct isoform-specific functions within cells (3–7). Protein kinase D isoforms are activated in response to numerous stimuli including reactive oxygen species (ROS), growth factors (i.e. platelet-derived growth factor), activators of G protein-coupled receptors, and triggering of immune cell receptors such as the B-cell receptor or T-cell receptor complexes (8–13). PKD activity is regulated by autoinhibition, membrane translocation, and activating phosphorylations (reviewed by Rozengurt et al. (14)). Several NH2-terminal protein domains of PKD such as the pleckstrin homology (PH) domain and the two C1 domains have negative regulatory, autoinhibitory functions and their deletion leads to constitutive activity of the enzyme (15, 16). Although the exact molecular mechanisms are not known, it was suggested that stimulus-mediated lipid binding to the C1 domains (5), protein binding to the PH domain (8), phosphorylations of serine residues of the activation loop in the kinase domain (17), or oxidative stress-mediated tyrosine phosphorylations in the PH domain of PKD release autoinhibition (18).

PKD1 is an important sensor for many inducers of oxidative stress such as Rotenone, diphenylethioniodinium, H2O2, per-vanadate, and L-buthionine-(S,R)-sulfoximine (10, 18–23). Importantly, PKD1 in response to activation by ROS leads to the induction of nuclear factor κB (NF-κB), a transcription factor that regulates SOD2 expression and cellular detoxification as well as cell survival (10, 19, 24). This signaling pathway could be of importance for processes regulating cell survival under oxidative stress conditions such as mechanisms that regulate aging or cancer. PKD2 also activates NF-κB in response to other stimuli such as BCR-Abl expression in myeloid leukemia cells and NF-κB, activated by this PKD isoform mediates interleukin 8 production in epithelial cells (25, 26).

An initial step in ROS-mediated PKD1 activation are tyrosine phosphorylations mediated by the kinases Src and Abl (10, 18, 21). It has been shown that Src-mediated phosphorylations in the PH domain lead to further activating phosphorylations, suggesting a conformational change as a first step in the PKD1 activation cascade (20, 27). PKD1 gains full activity after phosphorylation at two serine residues in the activation loop in the kinase domain, which is mediated by novel PKC (nPKC) isoforms. Four nPKC isoforms (PKCδ, PKCe, PKCη, and PKCθ) are known within cells and all have been described to directly phosphorylate the PKD1 activation loop serines (17, 20, 28, 29). However, depending on the cellular context or the activating stimulus there is specificity for one isoform over the other. For example, it has been shown that PKCe and PKCη activate PKD1 in response to growth factor signaling or at the Golgi (13, 17, 28, 30, 31), whereas only PKCδ regulates PKD1 activity in response to oxidative stress, Rho activation, or stimulation with angiotensin II (20, 23, 32). It was suggested that a nPKC/PKD activation complex is necessary to facilitate activation loop phosphorylation and full activation of PKD. The nPKC isoform PKCη, for example, associates with the PH domain of PKD1 in response to growth factor signaling (33). However, the sites of interaction of PKD with other novel PKC isoforms have not been mapped so far and it is particularly unclear how PKCδ interacts with PKD1 in response to ROS.

Recently, the C2 domain of protein kinase Cδ was described as a novel phosphotyrosine binding domain (34). In response to overexpression of Src this domain facilitates the interaction of PKCδ with the Src-binding glycoprotein CDCP1. The C2 domain interacts with a minimal phosphotyrosine consensus motif that was described as (V/I)-pY-(Q/R)-X-(Y/F)-X,
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whereby the tyrosine residue is phosphorylated by Src (34). Although additional proteins containing this motif such as MUC-1, Abl, and PLD2 all have been shown to associate with PKCδ, the experimental proof for interaction of these proteins with the C2 domain of PKCδ through this phosphorysine motif is still lacking (34). We here demonstrate that PKCδ interacts with PKD1, whereas a PKCδ lacking the C2 domain does not bind PKD1. This data suggests that the mechanism of how PKCδ binds to and activates PKD1 in response to oxidative stress is via its C2 domain. We describe a novel phosphorysine residue within a C2-binding motif in PKD1 that is phosphorylated by Src and regulates the interaction of PKD1 with PKCδ. We further show that this interaction is prerequisite for oxidative stress-mediated regulation of the PKD isoforms PKD1 and PKD2, but not for PKD3.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—HeLa cells were from the American Type Culture Collection and were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The anti-Abi, anti-PKCB, anti-PKCe, anti-PKCA, and anti-GST, and anti-PKD/ PKCu (C-20) antibodies were from Santa Cruz (Santa Cruz, CA), anti-Src from Upstate Biotechnology (Waltham, MA). Anti-HA and anti-FLAG were from Sigma. Anti-pS744/748 (recognizes the phosphorylated activation loop in PKD1/2/3) antibody was from BIOSOURCE/Invitrogen. The rabbit polyclonal pY95 antibody was raised against a KFPECGFpYGMYD-oligonucleotides as primer pairs for Y95Q mutation; 5′-gccgctcgagtcatcctgtacccacggaggagcc-3′ oligonucleotides as primer pairs for Y95F mutation; 5′-ctcccctagtttgcttcggagtaatgtaaga-3′ and 5′-gtccctcatctccgagcttacccga-3′ oligonucleotides as primer pairs for 15′-gttcttcagagttggcttcggagtaatgtaaga-3′ and 5′-aaacattccgaaaccacattcagggga-3′ oligonucleotides as primer pairs for Y95F mutation; 5′-ggtttcagagttggcttcggagtaatg-3′ and 5′-aattttgctcatacagaacccacactcaggaaa-3′ oligonucleotides were used as primer pairs with GST-PKD3 as a template to generate a GST-PKD3-F103Y expression construct. Wild-type GST-PKD3 and FLAG-PKD2 expression constructs have been obtained from Dr. V. Malhotra and Dr. T. Seufferlein and have been described elsewhere (6, 35). FLAG-tagged wild-type PKCδ and PKCδ-ΔC2 expression constructs were obtained by PCR using cyano fluorescent protein (CFP)-tagged wild-type PKCδ as template and 5′-ggggatccatgatcagagttggcttcggag-3′ and 5′-ggggatccatgatcagagttggcttcggag-3′ as primers and cloned via BamHI and Xhol into the expression vector pcDNA4/TO. Both CFP-tagged constructs were obtained from Dr. A. Newton (36). Constitutively active PKCδ was obtained from Dr. S. Ohno, constitutively active Abl from Dr. N. Rosenberg, and wild-type, dominant-negative (Src-V295R-Y527F) or constitutively active Src (Src-Y527F) from Dr. J. Brugge. All constructs were verified by DNA sequencing.

Immunoblotting, Immunoprecipitation, and GST Pull-down Assays—Cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, pH 7.4) plus Protease Inhibitor Mixture (Sigma). Lysates were used either for immunoblot analysis or proteins of interest were immunoprecipitated with the respective antibody (2 μg) followed by a 30-min incubation with protein G-Sepharose (Amersham Biosciences). Immunocomplexes were washed three times with ice-cold TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl), and resolved by SDS-PAGE. For GST pull-down assays lysates were incubated for 2 h with GST-Sepharose beads (Amersham Biosciences) and complexes were washed three times with ice-cold TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl), and resolved by SDS-PAGE.

Immunofluorescence—Cells were transfected (5 μg of DNA) and 24 h after transfection plated on glass coverslips at a density of 50,000 cells/well in a 24-well plate. The next day cells were washed twice with phosphate-buffered saline and fixed in 3.5% paraformaldehyde (15 min, 37 °C). Following permeabilization (0.1% Triton X-100, 10 min) cells were blocked with 3% bovine serum albumin and 0.05% Tween 200 in phosphate-buffered saline (blocking solution) for 30 min at room temperature. The coverslips were then incubated with primary antibody diluted in blocking solution anti-HA (rat), 1:2,000, overnight at 4 °C. Cells were then washed five times with phosphate-buffered saline.
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FIGURE 1. The NH2-terminal of PKD1 contains a PKCδ binding motif. A, nPKC isoforms and PKD1 were expressed in HeLa cells and cells were treated with hydrogen peroxide (10 min, 10 μM). PKD1 (anti-HA) was immunoprecipitated and samples were analyzed for co-immunoprecipitation of the respective nPKC isoform by immunoblotting. The nitrocellulose membrane was stripped and re-probed with anti-HA to control PKD expression (lower panel). B, domains of protein kinase D1. PKD1 consists of two zinc fingers, C1a and C1b, within the first 321 amino acids, an acidic region (AR), a pleckstrin homology domain (PH), and the kinase domain (KD). C, HA-tagged wild-type PKD1, or PKD1 deletion mutants (PKD1Δ1–321, PKD1ΔAR, PKD1ΔPH, or PKD1ΔΔKIN) were overexpressed in HeLa cells and cells were treated with hydrogen peroxide (10 min, 10 μM). PKD1 was immunoprecipitated (anti-HA) and samples were analyzed for co-immunoprecipitation of PKCδ by immunoblotting with anti-PKCδ (upper panel). The nitrocellulose membrane was stripped and re-probed with anti-HA to control PKD expression (lower panel).

Results

The NH2 Terminus of PKD1 Contains a PKCδ Binding Motif—PKD1 has important roles in the protective response to oxidative stress induced by hydrogen peroxide, where it promotes cellular survival and detoxification (37). H2O2 has been shown to lead to Src and PKCδ activation, which both in turn can phosphorylate PKD1 (19). In this activation mechanism Src causes priming phosphorylations that facilitate PKCδ-mediated phosphorylations, which lead to a fully active PKD enzyme (20, 27). However, the mechanisms of how PKCδ interacts with and activates PKD1 in response to oxidative stress are not known. First, we determined which nPKC isoforms besides PKCδ are capable to bind PKD1 in response to oxidative stress. We found that only the δ isoform binds to PKD1 after treatment of cells with H2O2 (Fig. 1A). Then, to map the region of interaction of both enzymes we analyzed the interaction of endogenous PKCδ with PKD1 deletion mutants after stimulation of cells with hydrogen peroxide. Therefore we expressed wild-type PKD1 or mutants deleted in the acidic region (ΔAR), the pleckstrin homology domain (ΔPH), the kinase domain (ΔKIN), and the NH2 terminus (Δ1–321) (Fig. 1, B and C). We found that in response to oxidative stress, PKCδ interacts within a region containing the first 321 amino acids of PKD1 (Fig. 1C). This NH2-terminal region of PKD1 contains the lipid-binding C1a and C1b domains as well as a putative transmembrane region and a 14–3–3 binding motif (38, 39). Within the NH2 terminus we also found a sequence containing two tyrosine residues (Phe94–Tyr95–Gly96–Met97–Tyr98–Asp99), similar to the recently described minimal consensus motif (V/I)-pY-(Q/R)-X-(Y/F)-X that facilitates interaction of proteins with the PKCδ C2 domain (Fig. 2A) (34). We mutated both tyrosines in this motif to phenylalanine (PKD1-Y95F; PKD1-Y98F) or to glutamine (PKD1-Y95Q; PKD1-Y98Q) and analyzed the interaction of these PKD1 mutants with PKCδ in response to oxidative stress (Fig. 2B). Interestingly, PKD1 mutants with tyrosine to phenylalanine or glutamine mutations at residue 95 lost their ability to interact with PKCδ suggesting that a tyrosine at this position is required. On the other hand a PKD1 mutant with tyrosine at residue Tyr98 mutated to phenylalanine was still capable of interacting with PKCδ in response to oxidative stress. However, a tyrosine 98 to glutamine PKD1 mutant (PKD1-Y98Q) lost its ability to interact with PKCδ, suggesting that position 98 allows tyrosine or phenylalanine, which concurs with the requirements of the consensus motif for PKCδ binding (Fig. 2A).

Oxidative Stress-mediated Phosphorylation of PKD1 at Tyrosine Residue 95—Tyr95 mediates binding of PKD1 to PKCδ in response to oxidative stress. Furthermore, the Tyr95–Gly–Met–Leu–Tyr–Phe sequence in PKD1 shows similarity to the minimal consensus sequence (V/I)-pY-(Q/R)-IX-(Y/F)-X for PKCδ-binding proteins. We next analyzed if PKD1 is phosphorylated at tyrosine residue 95 in this potential PKCδ C2 domain

saline and incubated with secondary antibody diluted 1:500 in blocking solution (donkey anti-rat IgG Alexa Fluor 488) for 2 h at room temperature. After extensive washes in phosphate-buffered saline coverslips were mounted in Fluormount-G (Southern Biotech, Birmingham, AL) and examined.

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binding motif. To analyze phosphorylation of this site in response to oxidative stress, we generated a phosphospecific antibody that specifically recognizes PKD phosphorylated at tyrosine residue 95 (anti-pY95). To demonstrate phosphorylation of tyrosine 95 in response to oxidative stress we transfected wild-type PKD1, PKD1-Y95F, and PKD1-Y98F mutants and analyzed the phosphorylation of Ty r95 after induction of oxidative stress. As expected, a PKD1-Y95F mutant was not phosphorylated in response to oxidative stress, whereas wild-type PKD1 and a PKD1-Y98F mutant were tyrosine phosphorylated and were recognized by the antibody (Fig. 3A). This experiment also indicates specificity of the pY95 antibody for the phosphorylated Tyr95 residue. Because a mutational analysis always bears the possibility that point mutants are mislocalized within cells, we analyzed cellular localization of the wild-type PKD1 and the Y95F mutant and found no significant differences in cellular localization either before or after hydrogen peroxide treatment (Fig. 3B). We also used the anti-pY95 antibody to analyze oxidative stress-treated cells for tyrosine phosphorylation of endogenous PKD1 and PKD2 at this residue and found that both were phosphorylated at this tyrosine residue in response to oxidative stress (Fig. 3C). Tyrosine phosphorylation of Tyr95 did not occur in response to treatment of cells with phorbol ester, which mimic diacylglycerol formation and thus activate PKC isoforms and PKD in some signaling pathways (supplemental Fig. 1).

Because we have shown in previous studies that tyrosine kinases Src and Abl are upstream of PKD1 in response to oxidative stress signaling and that both can directly phosphorylate certain tyrosine residues in PKD1, we next analyzed if the tyrosine phosphorylation of PKD1 at Tyr95 is mediated by these kinases. We expressed constitutive-active Abl or Src and analyzed if this leads to PKD1 phosphorylation at residue Tyr95 and found that Src, but not Abl mediates Tyr95 phosphorylation of overexpressed and endogenous PKD1 (Fig. 4, A and B). This is in accordance with Benes et al. (34), who identified Src as the kinase that leads to the phosphorylation of the tyrosine residue of the PKCδ binding motif of other targets that interacts with the C2 domain of PKCδ. Moreover, the inhibition of Src with PP2 led to an inhibition of oxidative stress-mediated phosphorylation of PKD1 at Tyr95, further indicating the importance of Src in this activation mechanism (Fig. 4C). Finally, to determine whether Src is the major regulator of PKD1 pY95 phosphorylation, we compared cells transfected with wild-type (WT-Src) and dominant-negative Src (DN-Src) and determined PKD1 Tyr95 phosphorylation in response to oxidative stress. Dominant-negative Src completely blocked PKD1 phosphorylation, indicating that Src (but not other Src-like kinases) is the mediator of PKD1 phosphorylation at pY95 (Fig. 4D). A more detailed analysis of how Src contrib-
utes to the phosphorylation of this site will be the subject of future studies.

PKCδ Interaction with PKD Isoforms Occurs via a pY-Gly-Met/Leu-Tyr Motif—We next analyzed the amino acid sequences of all three PKD isoforms, PKD1/PKCμ, PKD2, and PKD3/PKCδ for the potential PKCδ interaction motif. Interestingly, PKD1 and PKD2 contain YGMY (PKD1) or YGLY (PKD2) motifs, whereas PKD3 has a EGMY motif in the homologous region (Fig. 5A). According to our data showing the importance of phosphorylation of the first tyrosine residue (pY95 in PKD1) in the YG(M/L)Y motif, we hypothesized that PKD1 and PKD2 bind PKCδ, whereas PKD3 does not bind, due to a phenylalanine instead of a tyrosine at this position. We tested this by overexpressing tagged versions of all three PKD isoforms and stimulation of cells with H₂O₂. We then pulled down the PKD isoforms (immunoprecipitations or GST-Sepharose) and analyzed co-immunoprecipitation of PKCδ. As predicted, PKD1 and PKD2 interact with PKCδ, whereas PKD3 does not (Fig. 5B). However, the mutation of phenylalanine 103 in the EGMY motif in PKD3 to tyrosine (F103Y) generates a YGMY motif and facilitates interaction of PKD3 with PKCδ. The PKD3-F103Y mutant gains the ability to interact with PKCδ in response to H₂O₂, and is tyrosine phosphorylated at this residue as demonstrated with the pY95 antibody, further emphasizing that a phosphotyrosine at this position is necessary for the docking of PKCδ to PKD isoforms (Fig. 5C).

The C2 Domain of PKCδ Interacts with PKD1 in Response to Oxidative Stress—Because the PKCδ interaction motif in PKD1 is highly homologous to a minimal consensus binding motif for the C2 domain of PKD, we next tested if PKCδ indeed interacts with PKD1 through its C2 domain. Therefore, we expressed PKD1 together with wild-type PKD3 or a PKD3 mutant deleted in the C2 domain (PKD3ΔC2). We then stimulated cells with oxidative stress (H₂O₂) and either immunoprecipitated PKCδ (Fig. 6A) or PKD1 (Fig. 6B). Samples were analyzed for co-precipitation of PKD1 (Fig. 6A) or PKCδ (Fig. 6B), respectively. Oxidative stress-mediated interaction of PKD1 with PKCδ was abrogated in the C2 deletion mutant, indicating an interaction of the phosphotyrosine motif of PKD1 with the C2 domain of PKCδ.

Tyrosine Phosphorylation at Residue Tyr⁹⁵ Is Required for PKCδ-mediated PKD Activation—We have shown before that in response to oxidative stress (H₂O₂), PKD1 activation loop phosphorylations are mediated by the novel PKC isoform PKCδ (20). It has been shown in many studies that PKC-mediated activation loop phosphorylations at two serine residues (Ser⁷³⁸/Ser⁷⁴² for human PKD1) directly translate to PKD activity (17). We here compared all three PKD isoforms (PKD1, PKD2, and PKD3) as well as the PKD3-F103Y mutant (gain of interaction with PKCδ) for activation loop phosphorylations after overexpression of active PKCδ. We found that both, PKD1 and PKD2, as well as a PKD3-F103Y mutant are regulated by PKCδ, whereas wild-type PKD3 is not phosphorylated by this kinase (Fig. 7). This demonstrates that phenylalanine in the required phosphotyrosine position not only blocks PKCδ binding to PKD3, but also PKD3 activation-loop phosphorylation by PKCδ. The mutation of phenylalanine 103 in PKD3 to a tyrosine rescues PKD3 phosphorylation by PKCδ because this mutant generates a PKCδ interaction motif. This suggests that the presence of a pY-G-M-L-Y motif in PKD isoenzymes generally allows PKCδ binding and subsequently activation by this enzyme.

We next analyzed if the interaction of PKCδ with PKD1 is required for oxidative stress-mediated activation. To specify that interaction of PKCδ and PKD1 through the pY-G-M/L-
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Y/F motif is required for PKD activation, we compared wild-type PKD1 to PKD1-Y95F (no interaction with PKC8 possible) and PKD1-Y98F (control) mutants. Blocking the interaction of PKC8 with PKD1 (PKD1-Y95F mutant) resulted in an almost complete loss of PKD1 activation loop phosphorylation (Fig. 8A). We then compared time kinetics of oxidative stress-mediated phosphorylation of tyrosine residues 463 and 95 and activation loop serines 738 and 742 (Fig. 8B). Tyrosine 463 phosphorylation is detectable as early as 1 min after treatment of cells with H2O2. PKD1 Tyr95 phosphorylation showed 50% intensity after 2 min and reached a maximum after 4 min of H2O2 treatment. Activation loop phosphorylation showed 50% intensity after 4 min and reached maximum phosphorylation after 6 min. This demonstrates that tyrosine phosphorylation at Tyr463 occurs first and that phosphorylation at Tyr95 precedes activation loop phosphorylation. To further emphasize this multistep activation model for oxidative stress, we performed mutational analysis.

Sequential Order of Oxidative Stress-mediated Tyrosine Phosphorylations and PKC8 Binding—Tyrosine phosphorylation of PKD1 is prerequisite for its activation by oxidative stress. Particularly, the phosphorylation of a tyrosine residue within the autoinhibitory pleckstrin homology domain, tyrosine 463, has been implicated in PKD1 activation by inducing a molecular switch that facilitates activation loop phosphorylation by PKC8 (10, 20). However, it was equally clear from previous studies that additional tyrosine phosphorylation sites outside of the PH domain exist (18). We now have identified an additional tyrosine phosphorylation site in PKD1, tyrosine residue 95, that facilitates the binding of PKC8 to PKD1 upon induction of oxidative stress. To further understand the tyrosine phosphorylation events leading to H2O2-mediated PKD1 activation, we made use of a subset of PKD1 mutants that mimic tyrosine phosphorylations. We and others have described previously, that the mutation of tyrosine 463 to a glutamate mimics phosphorylation and leads to a conformational change that allows PKD1 activation loop phosphorylation (Fig. 8C, middle panel), which directly correlates with PKD1 activation (10, 20). Interestingly this PKD1-Y463E mutant is constitutively phosphorylated at Tyr95 (Fig. 8C, upper panel). This implicates that both, phosphorylation at Tyr95 and activation loop phosphorylation is a consequence of the phosphorylation at Tyr463. Furthermore, as predicted, a PKD1-Y463E mutant binds to PKC8, because it is phosphorylated at Tyr95 (Fig. 9A). We also found that the mutation of tyrosine residue 95 to a glutamate facilitates PKC8 binding to PKD1, suggesting that this is a mutation that mimics the phosphorylation of this site. Finally, a PKD1-Y95F/Y463E mutant was not able to bind PKC8, further supporting our proposed activation model (Fig. 9B).

Taken together, the data presented here further support a model of PKD1 activation by oxidative stress that is initiated by tyrosine phosphorylations in the PH domain (pTyr463), leading to a conformational change and PKD activation. We now show that additional tyrosine phosphorylations at Tyr95 are
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FIGURE 8. Tyrosine phosphorylation at residue Tyr95 is required for oxidative stress-mediated PKD1 activation. A, vector control, HA-tagged wild-type PKD1 or PKD1-Y95F and PKD1-Y98F mutants were transfected in HeLa cells and cells were treated with hydrogen peroxide (10 min, 10 mM). PKD1 was immunoprecipitated (anti-HA) and samples were analyzed for phosphorylation of activation loop serines 738 and 742 (anti-pS738/742). The nitrocellulose membrane was stripped and re-probed for PKD1 expression (anti-PKD). B, HA-tagged PKD1 was transfected in HeLa cells and cells were treated with hydrogen peroxide (10 min) in a time-dependent manner (0 – 8 min). PKD1 was immunoprecipitated (anti-HA) and samples were analyzed for phospho-
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required for PKD1 activation because it mediates PKCδ-PKD1 complex formation and allows PKCδ-mediated activation loop phosphorylation. A suggested sequential model of PKD1/2 activation mechanisms in response to oxidative

FIGURE 9. Interaction of PKCδ with PKD mutants. A, vector control, HA-tagged wild-type PKD1, PKD1-Y463E, or PKD1-Y95E mutants were transfected in HeLa cells and analyzed for PKCδ binding. Cells transfected with wild-type PKD1 and treated with hydrogen peroxide (10 min, 10 mM) served as control. The nitrocellulose was stripped and re-probed for PKD1 expression (anti-
PKD). Equal PKCδ input was controlled by immunoblotting. B, HA-tagged wild-type PKD1; PKD1-Y463F; PKD1-Y463E; or PKD1-Y95F/Y463E mutants were transfected in HeLa cells and analyzed for PKCδ co-immunoprecipitation. The nitrocellulose was stripped and re-probed for PKD1 expression (anti-PKD). Equal PKCδ expression was controlled by immunoblotting. All results are typical of three independent experiments.

DISCUSSION

The serine/threonine kinase protein kinase D1 was recently identified as a sensor for oxidative stress that is activated by mitochondria-generated and other ROS (10, 19, 21, 23). Oxidative stress-activated PKD1 augments cell survival by induction of the transcription factor NF-κB, resulting in the up-regulation of anti-apoptotic and antioxidant genes (37). The AGC kinase PKCδ plays a central role in the responses to several stresses such as genotoxic stress or oxidative stress and also regulates NF-κB and apoptotic cell death (27, 40, 41). In the

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**DISCUSSION**

The serine/threonine kinase protein kinase D1 was recently identified as a sensor for oxidative stress that is activated by mitochondria-generated and other ROS (10, 19, 21, 23). Oxidative stress-activated PKD1 augments cell survival by induction of the transcription factor NF-κB, resulting in the up-regulation of anti-apoptotic and antioxidant genes (37). The AGC kinase PKCδ plays a central role in the responses to several stresses such as genotoxic stress or oxidative stress and also regulates NF-κB and apoptotic cell death (27, 40, 41). In the
present study, we describe the molecular mechanism by which oxidative stress leads to PKCδ-mediated activation of PKD. We report the direct interaction of both enzymes, which is mediated by a previously unknown tyrosine phosphorylation site in PKD and the phosphotyrosine-binding C2-domain of PKCδ. We further show that this interaction is prerequisite for oxidative stress- and PKCδ-mediated PKD activation.

We found that in response to oxidative stress, PKCδ interacts with the NH₂-terminal region of PKD1 (Fig. 1). Within this region we identified an amino acid motif (Tyr95-Gly96-Leu97-Tyr98) that resembled a potential minimal phosphotyrosine binding site for PKCδ. It was shown recently that the C2 domain of PKCδ is a phosphotyrosine binding domain that has a strong preference for aromatic residues three amino acids COOH-terminal to a phosphorylated tyrosine (pY) and the sequence pY-Gln/Arg-X-Tyr/Phe was identified as a minimal optimal binding sequence (34). We analyzed both tyrosine residues (Tyr95 and Tyr98) in PKD1 for phosphorylations in response to oxidative stress and found that binding of PKD by the C2 domain of PKCδ requires phosphorylation of Tyr95 in the YGLY motif (Figs. 2–4). We first performed a mutational analysis to identify Tyr95 as a potential phosphorylation and PKCδ interaction site (Figs. 2 and 3). We then generated a phosphospecific antibody (anti-pY95) to demonstrate that oxidative stress-activated PKD1 is indeed phosphorylated at this residue. We analyzed Tyr95 phosphorylation of the PKD1 mutants PKD1-Y95F and PKD1-Y98F compared with wild-type PKD in response to oxidative stress (Fig. 3A). The mutation of tyrosine 98 to phenylalanine had no effect on PKCδ binding, which is in accordance with the requirements of the ideal motif described by Benes et al. (34) showing that the +3 position relative to the phosphotyrosine (pY95) can be a tyrosine or phenylalanine. Analysis of all three PKD isoforms, PKD1/PKCμ, PKD2, and PKD3/PKCν for the PKDδ-C2 domain binding motif revealed that the pY-X-X-Y/F motif is also present in PKD2, but not in PKD3, which has a phenylalanine instead of a tyrosine residue at the relevant position (Fig. 5A). Consequently PKD3 was not able to bind PKCδ in response to oxidative stress (Fig. 5B).

The mutation of the phenylalanine to a tyrosine at position 103, however, restored the motif (FGMY to YGMY) in PKD3 and facilitated oxidative stress-mediated tyrosine phosphorylation of this residue as well as binding of PKCδ (Fig. 5C). This is particularly interesting because it suggests a function for PKD1 and -2, but not for PKD3 in oxidative stress signaling, where PKCδ is the upstream kinase for PKD (20). PKD isoforms have overlapping functions as demonstrated for anterograde membrane trafficking (42) and Golgi transport (2, 6). However, a specific function for PKD3 in basal glucose transport has been described (3). Furthermore, specific functions for PKD1 and -2 isoforms were identified recently for mechanisms regulating protein trafficking (43). We here demonstrate an elusive role for PKD1 and -2 in oxidative stress signaling, not shared by PKD3 as it is not tyrosine phosphorylated (Fig. 5C) nor activated by PKCδ (Fig. 7). Therefore this is one of the first studies demonstrating distinct, non-overlapping functions of PKD isoforms.

Oxidative stress-mediated tyrosine phosphorylations of PKD are mediated by Src and Abl (10, 18). Interestingly, all so far described phosphorylations (i.e. at Tyr463) that are mediated by these tyrosine kinases occur in the autoinhibitory pleckstrin homology domain of PKD1 and it was suggested that they induce a conformational change that allows activation (18). An important question therefore is, if the Tyr95 phosphorylation is a consequence of the Tyr463 phosphorylation. To answer this, we utilized a previously described PKD1 mutant (PKD1-Y463E) that mimics phosphorylation of this site and is constitutively phosphorylated at the activation loop serines and active (20). Interestingly, this mutant is also phosphorylated at Tyr95 (Fig. 8C), suggesting that Tyr95 phosphorylation is a consequence of Tyr463 phosphorylation.

A Scansite analysis (www.scansite.mit.edu/) for phosphorylation motifs revealed tyrosine 95 as a potential Src phosphorylation site and we used the anti-pY95 antibody and a subset of tools (Src inhibitor, dominant-negative, or constitutively active Src) to demonstrate, that this tyrosine residue is indeed phosphorylated by Src (Fig. 4). This is particularly interesting because PKCδ and PKD under activating conditions both have been shown to associate with active Src by a so far unidentified mechanism (18, 44), suggesting a PKD activation complex composed of these three kinases.

The importance of PKCδ in oxidative stress-mediated activation of PKD1 has been described previously (20, 27). It was shown that PKCδ is activated via tyrosine phosphorylations, mediated by Src family kinases (45), and that in response to ROS Src is upstream of PKCδ (20, 46, 47). PKCδ is known to bind several proteins including actin, GDCP1, and GAP-43 via its C2 domain (34, 48, 49). It was recently shown for the Src-associated CDCP1 protein that phosphorylation by Src also generates a phosphomotif that mediates the interaction with the PKCδ C2 domain (34). We here suggest a similar mechanism, Src-mediated phosphorylation of PKD1 that leads to the generation of a PKCδ binding site and eventually PKD1 activation.

Taken together, our results identify a novel tyrosine phosphorylation motif in PKD1 and PKD2. The phosphorylation of this motif in response to oxidative stress is crucial for the inter-

**FIGURE 10. Model of the suggested molecular mechanisms leading to H₂O₂-mediated PKD activation.** In response to oxidative stress Src mediates activation of Abl which in turn directly phosphorylates PKD in the PH domain at tyrosine residue 463 (step 1) (shown in Refs. 10 and 20). This leads to a conformational change (step 2) that allows Src-mediated phosphorylation of PKD at tyrosine residue Tyr95 in the Tyr-Gly-Leu/Met-Tyr/Phe motif (step 3). Phosphorylation of this site facilitates the C2-domain of PKCδ to form an activation complex with PKD that mediates PKCδ-induced phosphorylation of PKD in the activation loop at serines 738 and 742 (step 4), resulting in a fully active PKD enzyme.
action of both kinases with the C2 domain of PKCδ, a recently
described novel phosphotyrosine binding domain. With the
data presented here we not only identify PKD1 and PKD2 as
novel PKCδ interaction partners, but also show that this inter-
action eventually leads to PKCδ-mediated phosphorylation and
activation.

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