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Protein Kinase D2 Mediates Activation of Nuclear Factor κB by Bcr-Abl in Bcr-Abl$^+$ Human Myeloid Leukemia Cells

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ABSTRACT

The Bcr-Abl tyrosine kinase activates various signaling pathways including nuclear factor κB that mediate proliferation, transformation, and apoptosis resistance in Bcr-Abl$^+$ myeloid leukemia cells. Here we report that protein kinase (PK) D2, a serine threonine kinase of the PKD family, is a novel substrate of Bcr-Abl. PKD2 was found to be the major isoform of the PKD family expressed in chronic myeloid leukemia cells and is tyrosine phosphorylated by Bcr-Abl in its pleckstrin homology domain. A mutant that mimicks tyrosine phosphorylation of PKD2 in the pleckstrin homology domain activates nuclear factor κB independently of its catalytic activity. Furthermore, our data show that Bcr-Abl–induced activation of the nuclear factor κB cascade in LAMA84 cells is largely mediated by tyrosine-phosphorylated PKD2. These data present a novel mechanism of Bcr-Abl–induced nuclear factor κB activation in myeloid leukemia. Targeting PKD2 tyrosine phosphorylation, not its kinase activity, could be a novel therapeutic approach for the treatment of Bcr-Abl$^+$ myeloid leukemia.

INTRODUCTION

Chronic myelogenous leukemia (CML) results from the expression of the Bcr-Abl oncogene. This gene encodes a fusion protein that is found in the cells of 95% of patients with CML and renders the kinase constitutively active. The activity of the Bcr-Abl tyrosine kinase confers a survival advantage to hematopoietic cells, contributes to leukemic transformation, and makes the cells less sensitive to apoptosis induced by cytotoxic drugs. Multiple signaling/survival pathways have been implicated in this phenomenon, including dysregulation of nuclear factor (NF)-κB. Indeed, Bcr-Abl was found to induce activation of the NF-κB pathway in Bcr-Abl$^+$ CML cells (1, 2). MEKK1 and p38^{mapk} have been implicated in Bcr-Abl–induced NF-κB activation (3, 4), and there is an intense search for additional signaling pathways that mediate this effect. Recently, a new family of serine/threonine protein kinases (PKs) has been identified that comprises three members, PKD1 (also termed PKCμ; refs. 5 and 6), PKD2 (7), and PKD3 (also named PKCν; ref. 8). PKD1 has been the focus of most studies to date. This kinase is implicated in multiple biological processes, such as proliferation, invasion, regulation of the Golgi apparatus, and apoptosis. PKDs can be activated by phorbol esters or via the activation of G protein-coupled receptors and members of the PKC family and regulate multiple intracellular signaling pathways (9). Recent data show that PKD1 potentiates NF-κB activation in response to oxidative stress. This effect is mediated by Src and Abl tyrosine kinases and requires tyrosine phosphorylation in the pleckstrin homology (PH) domain at Tyr^{463} as well as the catalytic activity of PKD1 (10, 11).

Here we examined the role of PKDs in Bcr-Abl–induced NF-κB activation in Bcr-Abl$^+$ LAMA84 human myeloid leukemia cells. Our data show that PKD2 is the major PKD isoform expressed in LAMA84 cells. PKD2 is constitutively tyrosine phosphorylated in these cells by Bcr-Abl and mediates Bcr-Abl–induced NF-κB activation. In contrast to PKD1, NF-κB activation by tyrosine-phosphorylated PKD2 is independent of PKD2 catalytic activity, suggesting that PKD2 acts as a docking protein and not as a kinase to mediate NF-κB activation. Thus, the tyrosine-phosphorylated PH domain of PKD2, but not its kinase activity, may represent a novel target for antileukemic strategies.

MATERIALS AND METHODS

Site-Directed Mutagenesis. All PKD2 expression plasmids are based on an NH_2-terminal FLAG-PKD2 in pcDNA3, derived from full-length human PKD2 (7). Mutagenesis was carried out by polymerase chain reaction using QuikChange XL site-directed mutagenesis as described by the manufacturer (Stratagene, La Jolla, CA) with the following primer pairs: PKD2-D^{695}A, 5′-caggtgaagctgtgtgcctttggctttgctcgc-3′ and 5′-gcgagcaaagccaaaggcacacagcttcacctg-3′; PKD2-S$^{706/710}$E, 5′-catcggcgagaaggagttccgcgaagtggtgggcacgccg-3′ and 5′-cggcgtgcccaccacttcgcggcggaactccttctcgccgat-3′; PKD2-Y^{438}D, 5′-aacacgaccaacagatacgacaaggaaattccgc-3′ and 5′-gcggaatttccttctggtatctgttggtcgtgttg-3′; PKD2-Y^{438}F, 5′-caacacgaccaacagatactttaaggaaattccgc-3′ and 5′-gcggaatttccttaaagtatctgttggtcgtgttg-3′; and PKD2-ΔPH, 5′-aaatccagccccgtcatccttcag-3′ and 5′-gaataggcccctctagatgcatgc-3′. PKD2-Y^{438}D/D^{695}A and PKD2-Y^{438}D/S$^{706/710}$E were generated from the PKD2-D^{695}A and PKD2-S$^{706/710}$E mutants, respectively. All constructs were verified by DNA sequencing.

Immunoblotting and Immunoprecipitation. Cells were lysed in a buffer containing 50 mmol/L Tris/HCl (pH 7.4), 1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and a protease inhibitor mixture (Sigma, Deisenhofen, Germany). Lysates were used for immunoblot analysis or subjected to immunoprecipitation by a 1-hour incubation with the respective antibody, followed by a 30-minute incubation with protein A-Sepharose (AP Biotech, Freiburg, Germany). Immune complexes were resolved by SDS-PAGE or subjected to *in vitro* kinase assays.

Immune Complex Kinase Assays. The IKK assays were performed essentially as described previously (12) using recombinant glutathione *S*-transferase (GST)-IκB-(1–54) as substrate. Autokinase activity of FLAG-tagged PKD2 expression plasmids was determined as described previously (13). For the Bcr-Abl *in vitro* kinase assay, recombinant PKD2 in kinase buffer [30 mmol/L Tris/HCl (pH 7.4), 30 mmol/L $MgCl_2$, 2 mmol/L dithiothreitol, and 100 μmol/L ATP] was incubated with Bcr-Abl immunoprecipitated from LAMA84 cells in the presence or absence of STI-571 at 30°C for various times as indicated. The reaction was stopped by adding 6× sample buffer, and PKD2 tyrosine phosphorylation was analyzed by SDS-PAGE followed by anti-Tyr(P) Western blotting.

Cell Transfection and Reporter Gene Assays. LAMA84 cells were electroporated in 300 μL of media at 280 V using 5 μg of a NF-κB firefly luciferase reporter plasmid, 10 ng of a *Renilla* luciferase reporter plasmid, and 5 μg of the various pcDNA3-FLAG-PKD2 expression plasmids as indicated. Twenty-four hours after electroporation, cells were collected by density gradient centrifugation for 20 minutes at 1,400 rpm. Exponentially growing HEK293 cells (5×10^4 cells per 35-mm dish) were transfected with various pcDNA3-FLAG-PKD2 expression plasmids, the NF-κB firefly luciferase re-

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Note: T. Mihailovic and M. Marx contributed equally to this work.

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porter, and the *Renilla* luciferase reporter plasmid using FuGENE 6 as described in detail by the manufacturer (Roche Diagnostics, Mannheim, Germany). Twenty four hours after transfection, cells were incubated with 500 nmol/L H_2O_2 for another 16 hours as indicated. The activities of firefly and *Renilla reniformis* luciferase in the dual luciferase reporter assay were determined as described by the manufacturer (Promega, Madison, WI).

Electrophoretic Mobility Shift Assay and Supershift Analysis. Nuclear protein extracts were prepared and incubated with a ^{32}P-labeled double-stranded oligonucleotide probe corresponding to the κB motif of the mouse immunoglobulin light chain enhancer (5′-AGCTTGGGGACTTTCCACTAG-TACG-3′) as described previously (12). DNA protein complexes were resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel in Tris/glycine/EDTA buffer. Dried gels were subjected to X-ray film with intensifying screens. For competition experiments, unlabeled double-stranded oligonucleotides were added to the binding reactions in approximately 20-fold molar excess. For supershift analysis, antibodies against RelA (p65), RelB, c-Rel, p50, and p52 (4 μg of each per reaction; Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction buffer. After 30 minutes on ice, the labeled probe was added, and the reaction was performed at room temperature for 15 minutes. Samples were analyzed as described above.

Cells and Materials. HEK293 cells were from the American Type Culture Collection (Manassas, VA), and LAMA84 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). STI-571 was a kind gift from Novartis Pharma AG (Basel, Switzerland) and prepared as a 10 mmol/L stock solution in dimethyl sulfoxide (Sigma). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and the protein kinase inhibitor GF109203X were obtained from Sigma. Anti-Abl and anti–phospho-Abl antibodies were from Cell Signaling (NEB, Frankfurt, Germany). Antibodies directed against PKCμ/PKD1, IKK, RelA (p65), RelB, c-Rel, p50, and p52 were from Santa Cruz Biotechnology, anti-PKD2 was from Orbigen (San Diego, CA), and the anti-Tyr(P) antibody (clone 4G10) was from Upstate Biotechnology, Inc. (Waltham, MA). The anti-pSer^{876} PKD2 antibody has been described previously (7). [^{32}P]ATP (5000 Ci/mmol; 37 GBq = 1 mCi) was from Amersham Buchler GmbH (Braunschweig, Germany). All other reagents were from standard suppliers as indicated in the text. For statistical analysis, the *t* test for nonpaired samples was used.

RESULTS

Protein Kinase D2 Is the Major Protein Kinase D Isoform in Human LAMA84 Cells. The fusion of Bcr to the Abl tyrosine kinase leads to constitutive activation of the enzyme. Indeed, endogenous Bcr-Abl exhibits constitutive tyrosine phosphorylation in LAMA84 cells that is markedly reduced by the selective Abl inhibitor STI-571 (Fig. 1*B*).

It has been demonstrated that another member of the PKD family, PKD1, is a downstream target of Abl on activation by oxidative stress (10, 11). To determine whether and which PKDs could mediate signaling induced by constitutively active Bcr-Abl, we first determined the PKD isoforms expressed in LAMA84 cells. Lysates were subjected to Western blot analysis using antibodies directed against PKD1 or PKD2 and compared with lysates of HEK293 cells transfected with PKD1 and PKD2 expression plasmids, respectively. An antibody directed against the COOH terminus of PKD1 that cross-reacts with PKD2 detected overexpressed PKD1 at a molecular weight of 115,000 as well as overexpressed PKD2 at a molecular weight of 105,000 to a similar degree as reported previously (7). In LAMA84 cells, only one major band corresponding to the molecular weight of PKD2 was detectable. These data were confirmed using an antibody that predominantly detects PKD2 and, to a lesser degree, overexpressed PKD1 (Fig. 1*C*). In addition, we examined another human myeloid leukemia cell line, K562, as well as primary myeloid cells from patients with Bcr-Abl$^+$ CML. All samples exhibited a major band corresponding to the molecular weight of PKD2 (Fig. 1*C*). These data suggest that PKD2 is the major PKD isoform expressed in Bcr-Abl$^+$ myelogenous leukemia cells.

Protein Kinase D2 Is a Novel Substrate of Bcr-Abl in LAMA84 Cells. Next we examined whether PKD2 was a potential substrate of Bcr-Abl in LAMA84 cells. As shown in Fig. 1*D*, PKD2 was found to be constitutively phosphorylated at tyrosine residues in LAMA84 cells. Incubation of the cells with the selective Bcr-Abl inhibitor STI-571 led to a marked reduction of PKD2 tyrosine phosphorylation, suggesting that tyrosine phosphorylation of PKD2 in LAMA84 cells is regulated by constitutively active Bcr-Abl. In contrast, treatment of LAMA84 cells with GF109203X, which blocks activation of PKD2 by members of the PKC family (13), had no effect on PKD2 tyrosine phosphorylation (Fig. 1*D*). Incubation of cells with TPA, which activates PKD2 by both binding to its cysteine-rich domain and activating members of the PKC family (13), led to an increase in phosphorylation of PKD2 at Ser^{876}, which can be used to monitor PKD2 kinase activity (7). However, TPA treatment had no effect on tyrosine phosphorylation of the kinase (Fig. 1*D*). Conversely, tyrosine phosphorylation of PKD2 did not correlate with an increase in phosphorylation at Ser^{876}, demonstrating that tyrosine-phosphorylated PKD2 is not constitutively active in LAMA84 cells.

To determine whether PKD2 was a direct substrate of Bcr-Abl, we performed *in vitro* kinase assays using recombinant PKD2 and Bcr-Abl immunoprecipitated from LAMA84 cells. As shown in Fig. 1*E*, Bcr-Abl directly induced tyrosine phosphorylation of PKD2 that was abolished in the presence of the STI-571. Thus, PKD2 is indeed a novel, direct substrate of Bcr-Abl.

Bcr-Abl Induces Activation of Nuclear Factor κB in LAMA84 Cells. Bcr-Abl can induce activation of the NF-κB pathway in Bcr-Abl$^+$ cells (1, 2). To determine whether this mechanism was functional in LAMA84 cells, NF-κB activity was examined by electrophoretic mobility shift assay (EMSA). Bandshift assays demonstrated constitutive activation of NF-κB in LAMA84 cells (Fig. 2*A*). NF-κB activity was further enhanced by treatment of cells with tumor necrosis factor (TNF)-α and was decreased in the presence of the Bcr-Abl inhibitor STI-571. In contrast, the PKC inhibitor had no effect on NF-κB activity in these cells. These data were confirmed by assaying the catalytic activity of the endogenous IKK complex. As shown in Fig. 2*B*, LAMA84 cells display elevated IKK activity that is enhanced by TNF-α but can be abolished by the Bcr-Abl inhibitor STI-571. A PKC inhibitor had no effect on IKK activity in LAMA84 cells. This demonstrates that Bcr-Abl induces activation of NF-κB also in LAMA84 cells.

To further analyze the composition of NF-κB complexes in LAMA84 cells, supershift assays were performed. Fig. 3*A* demonstrates that antibodies directed against p52 and p65 lead to a reduction of basal DNA binding. This effect is especially pronounced on addition of the p52 antibody. In contrast, no effect on DNA binding was observed after adding antibodies against p50, RelB, or c-Rel (Fig. 3). This indicates that NF-κB complexes in LAMA84 cells are mainly composed of p52 and p65 subunits. TNF-α treatment of LAMA84 cells led to a clear supershift after adding p65 and p50 antibodies. No supershift was observed after treatment with the Bcr-Abl inhibitor STI-571 (Fig. 3).

Protein Kinase D2 Mediates Abl-Induced Nuclear Factor κB Activation in Response to Oxidative Stress. Having established that Bcr-Abl induces tyrosine phosphorylation of PKD2 and activation of NF-κB in LAMA84 cells, we asked whether PKD2 could be a mediator of Bcr-Abl–stimulated NF-κB activation. It has been shown that activation of Abl by oxidative stress leads to phosphorylation of PKD1 at Tyr^{463} in the PH domain, increased catalytic activity of PKD1, and subsequent activation of NF-κB (10, 11). Therefore, we first examined whether PKD2 could activate NF-κB under similar conditions as PKD1 using a well-established model cell line, HEK293. H_2O_2 treatment of HEK293 cells transfected with wild-type

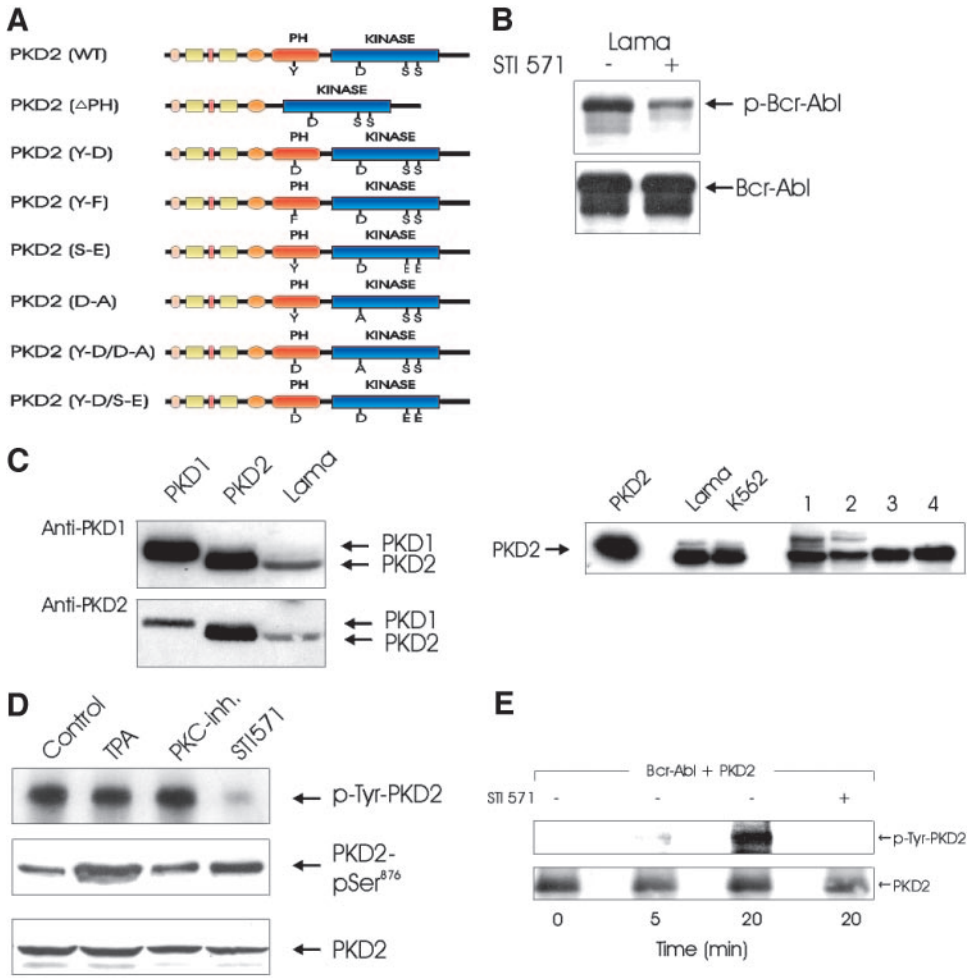

Fig. 1. *A*, PKD2 mutants used in this study. PKD2 mutants were generated as described in Materials and Methods. *PKD2 (WT)*, wild type; *PKD2 (ΔPH)*, mutant lacking the PH domain; *PKD2 (Y-D)*, Y-D exchange at residue 438; *PKD2 (Y-F)*, Y-F exchange at residue 438; *PKD2 (S-E)*, S-E exchange at residues 706 and 710; *PKD2 (D-A)*, D-A exchange at residue 695. *PKD2 (Y-D/D-A)* and *PKD2 (Y-D/S-E)* are double mutants at the residues stated above. *B*. Bcr-Abl is constitutively active in LAMA84 cells. Cells were treated with 250 nmol/L STI-571 for 48 hours (+) or received an equivalent amount of solvent (−). Lysates were subsequently analyzed by Western blotting using specific antibodies against Bcr-Abl and active, phosphorylated Bcr-Abl (*p-Bcr-Abl*) as indicated. *C*, expression of PKD1 and PKD2 in LAMA84 cells. *Left panels*, LAMA84 cells were lysed and analyzed by Western blotting with antibodies directed against PKD1 or PKD2 as indicated by *arrows*. As a control, HEK293 cells were transiently transfected with pcDNA3-PKD1 (*PKD1*) and pcDNA3-PKD2 (*PKD2*), respectively, and overexpressed PKDs were detected using the same antibodies as indicated by *arrows*. *Right panel*, HEK293 cells transfected with PKD2, LAMA84 cells, K562 cells, and four different samples of primary CML cells (*Lanes 1–4*) were lysed and analyzed by Western blotting using an antibody that primarily detects PKD2 as indicated by the *arrow*. *D*, constitutive tyrosine phosphorylation of PKD2 in LAMA84 cells. Cells were exposed to 250 nmol/L STI-571 for 48 hours or to the selective PKC inhibitor GF109203X (3.5 μmol/L) for 1 hour (*PKC-inh.*) or treated with 400 nmol/L TPA for 30 minutes. Control cells received an equivalent amount of solvent (*Control*). For detection of tyrosine-phosphorylated PKD2, cells were lysed and immunoprecipitated with anti-PKD2 antibody followed by anti-Tyr(P) Western blotting (*top panel, p-Tyr-PKD2*). Active PKD2 was monitored using an anti-$pSer^{876}$-PKD2 antibody (*middle panel*, *PKD2-$pSer^{876}$*). PKD2 expression was determined using the selective anti-PKD2 antibody (*bottom panel*). *E*, *in vitro* phosphorylation of PKD2 by Bcr-Abl. Recombinant PKD2 was incubated with Bcr-Abl immunoprecipitated from LAMA84 cells for the times indicated and subsequently analyzed by SDS-PAGE followed by anti-Tyr(P) Western blotting. The position of tyrosine-phosphorylated PKD2 is indicated by an *arrow* (*p-Tyr-PKD2*). The membrane was subsequently stripped and reprobed with an anti-PKD2 antibody (*PKD2*).

PKD2 induced tyrosine phosphorylation of PKD2, which was markedly reduced by STI-571 (Fig. 4*A*). Thus, H_2O_2-induced activation of Abl stimulates tyrosine phosphorylation of PKD2. A PKD2 mutant lacking the PH domain (ΔPH) exhibited virtually no tyrosine phosphorylation in response to H_2O_2, indicating that Abl induces tyrosine phosphorylation of PKD2 mainly in the PH domain. PKD1 is phosphorylated at Tyr^{463} in response to H_2O_2. This Tyr is positioned in a highly conserved region of the PKD family PH domain. Therefore, we replaced the Tyr residue corresponding to Tyr^{463} in PKD1, Tyr^{438} in PKD2, with Phe. This PKD2 mutant (Y-F) exhibited a lower level of tyrosine phosphorylation in response to H_2O_2 compared with wild-type PKD2. Furthermore, the residual tyrosine phosphorylation of the PKD2-Y438F mutant was not reduced by STI-571 treatment (Fig. 4*A*). Thus, Tyr^{438} is the major Tyr residue in PKD2 that is phosphorylated by Abl.

Next we examined whether PKD2 could activate NF-κB. Wild-type PKD2 had no effect on the activity of a NF-κB luciferase reporter in the absence of H_2O_2. However, when wild-type PKD2-transfected HEK293 cells were treated with H_2O_2, there was a marked increase in NF-κB activity (Fig. 4*B*). H_2O_2 induces tyrosine phosphorylation of PKD2 at Tyr^{438} via Abl (Fig. 4*A*). To determine whether phosphorylation of PKD2 at Tyr^{438} was sufficient to induce activation of NF-κB, we generated a PKD2 mutant that mimicks phosphorylation of PKD2 at this site, PKD2-Y438D. As shown in Fig. 4*B*, this mutant markedly stimulated NF-κB reporter activity, even in the absence of H_2O_2, and its effect on NF-κB reporter activity could not be further enhanced by oxidative stress. Conversely, PKD2-Y438F, a mutant that cannot be phosphorylated at Tyr^{438}, failed to induce NF-κB activation in both the absence and presence of H_2O_2 (Fig. 4*B*). These data demonstrate that tyrosine-phosphorylated PKD2 is a novel mediator of H_2O_2/Abl-induced NF-κB activation. Tyr^{438} in the PH domain of PKD2 is the major Tyr residue that is phosphorylated by

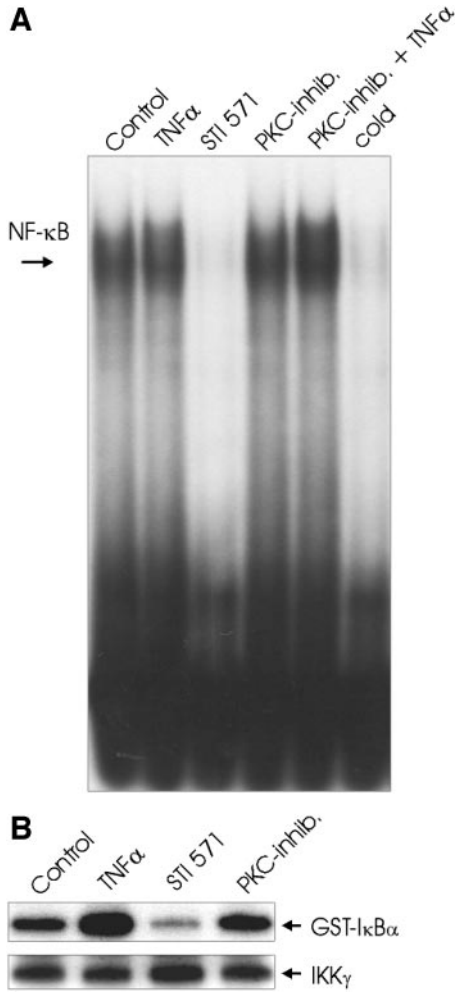

Fig. 2. *A*, electromobility shift assay in LAMA84 cells. Cells were incubated with either TNF-α (25 ng/mL, 20 minutes), STI-571 (250 nmol/L, 48 hours), PKC inhibitor (*PKC-inhib.*; 3.5 μmol/L, 1 hour), PKC inhibitor (3.5 μmol/L, 1 hour) followed by TNF-α (25 ng/mL, 20 minutes; *PKC-inhib. + TNFα*), or an equivalent amount of solvent (*Control*). The EMSA was performed as described in Materials and Methods. *Cold* represents competition with an excess of unlabeled oligonucleotide. The *arrow* indicates specific binding to the oligonucleotide probe. The blot represents one of three independent experiments. *B*, activation of endogenous IKKα/β in LAMA84 cells. Cells received solvent (*Control*) or were treated with TNF-α (25 ng/mL, 20 minutes), STI-571 (250 nmol/L, 48 hours), or PKC inhibitor (*PKC inhib.*; 3.5 μmol/L, 1 hour), respectively. The *top panel* shows the activity of endogenous IKK complex, measured by *in vitro* phosphorylation of recombinant GST-IκBα (amino acids 1–54). The *arrow* indicates phosphorylated GST-IκBα. Note the high basal activity of IKK and inhibition in the presence of STI-571. *Bottom panel,* samples were reprobed with anti-IKKγ antibody and visualized by enhanced chemiluminescence.

activated Abl, and tyrosine phosphorylation at this site is required for the induction of NF-κB activity by PKD2.

Protein Kinase D2-Induced Activation of Nuclear Factor κB Is Independent of Its Kinase Activity. Tyrosine phosphorylation of PKD1 and activation of NF-κB by this PKD isoform have been linked to an increased catalytic activity (11). Surprisingly, the PKD2-Y438D mutant stimulated NF-κB reporter activity (Fig. 4*B*) but did not exhibit a marked increase in kinase activity compared with wild-type PKD2 (Fig. 5*A*). In contrast, replacement of Ser^{706} and Ser^{710} in the activation loop of PKD2 (13) by Glu (PKD2-S706/710E) resulted in a striking increase in kinase activity. Similarly, a PKD2-Y438D/S706/710E double mutant exhibited a markedly enhanced kinase activity compared with wild-type PKD2 and the PKD2-Y438D mutant, respectively. Replacement of Asp^{695} in the DFG motif of the kinase by Ala (PKD2-D695A; Fig. 1*A*) rendered the kinase inactive. Consequently, a PKD2-Y438D/D695A double mutant exhibited virtually no basal autokinase activity (Fig. 5*A*).

To determine whether kinase activity was required for PKD2-induced NF-κB activation, we examined the effect of the kinase-inactive PKD2-D695A mutant on NF-κB reporter activity. As shown in Fig. 5*B*, PKD2-D695A alone had no effect on NF-κB activity but exhibited an effect similar to that of wild-type PKD2 on NF-κB reporter activity in the presence of H_2O_2. Furthermore, the PKD2-Y438D/D695A double mutant, which mimicks constitutive phosphorylation of PKD2 at Tyr^{438} but lacks any catalytic activity, induced NF-κB reporter activity to the same degree as the catalytically active PKD2-Y438D mutant (Figs. 4*B* and 5*B*) independently of H_2O_2.

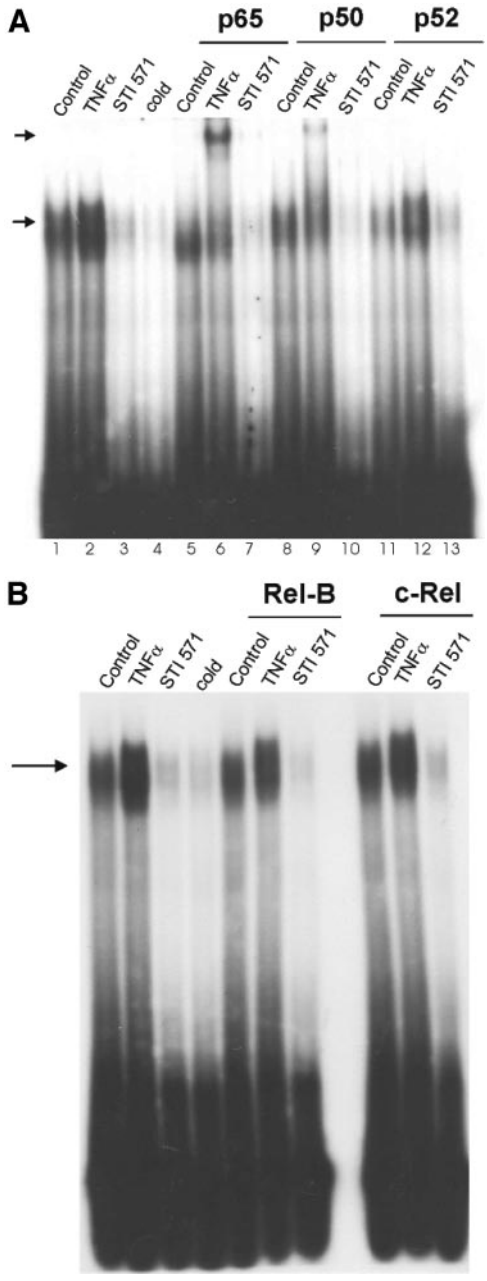

Fig. 3. Supershift analysis of the NF-κB complex in LAMA84 cells. Cells were incubated with TNF-α (25 ng/mL, 20 minutes), STI-571 (250 nmol/L, 48 hours), or an equivalent amount of solvent (*Control*). EMSA (*Lanes 1–4*) and supershift assays (*A*, *p65*, *p50*, and *p52*; *B*, *RelB* and *c-Rel*) were performed as described in Materials and Methods. *Cold* represents competition with an excess of unlabeled oligonucleotide. The *bottom arrow* indicates specific binding to the oligonucleotide probe, the *top arrow* shows the supershift after adding antibodies (p65, p50, p52, RelB, and c-Rel) as indicated.

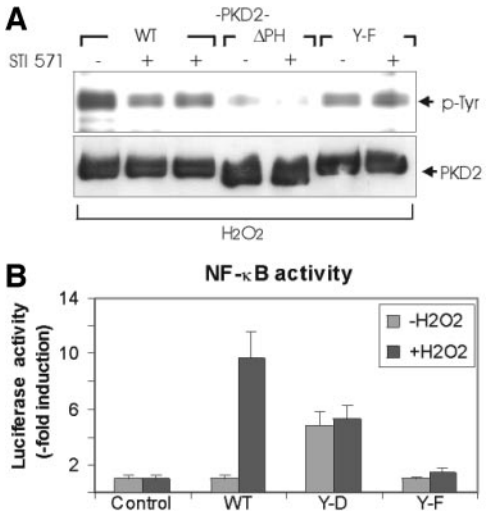

Fig. 4. *A*. Oxidative stress induces tyrosine phosphorylation within the PH domain of PKD2 via activation of Abl. HEK293 cells were transiently transfected with PKD2 wild-type (*WT*), PKD2-ΔPH (Δ*PH*), or PKD2-$Y^{438}F$ (*Y-F*); subsequently treated with STI-571 (5 μmol/L, 1 hour); and then incubated with 250 nmol/L H_2O_2 for 1 hour. Tyrosine phosphorylation of FLAG-tagged PKD2 or its mutants was determined by anti-FLAG immunoprecipitation followed by Western blotting using the monoclonal 4G10 antibody. The position of tyrosine-phosphorylated PKD2 is indicated by an *arrow* (*p-Tyr*). Blots were stripped and subsequently reprobed with the anti-PKD2 antibody (*arrow*, *PKD2*). *B*. PKD2 potentiates NF-κB activation in response to oxidative stress. HEK293 cells were cotransfected with a NF-κB firefly luciferase reporter plasmid, a control *Renilla* luciferase plasmid, and the respective PKD2 expression plasmids (wild-type PKD2, *WT*; PKD2-Y438D, *Y-D*; PKD2-Y438F, *Y-F*) for 16 hours and subsequently incubated with 250 nmol/L H_2O_2 for another 24 hours. NF-κB reporter assays were performed as described. All results are representative of three independent experiments, each performed in duplicates.

Thus, the kinase activity of PKD2 is not required for its effect on NF-κB activation. Accordingly, the constitutively active PKD2-S706/710E mutant had no effect on NF-κB reporter activity. Surprisingly, this mutant even failed to increase NF-κB activity in the presence of H_2O_2. Furthermore, the constitutively active PKD2-Y438D/S706/710E double mutant was not able to induce NF-κB reporter activity (Fig. 5*B*). Thus, maximum catalytic activity of PKD2 is not required and may even prevent the stimulatory effect of the enzyme on NF-κB activity.

Tyrosine-Phosphorylated Protein Kinase D2 Mediates Nuclear Factor κB Activation by Constitutively Active Bcr-Abl in LAMA84 Cells. The data above established that in contrast to PKD1, PKD2 mediates NF-κB activation in response to active Abl in the absence of its catalytic activity. Because PKD2 is tyrosine phosphorylated but not constitutively active in LAMA84 cells, we next examined whether tyrosine-phosphorylated PKD2 could mediate constitutive NF-κB activity by Bcr-Abl in these cells. Wild-type PKD2 had no major effect on basal NF-κB reporter activity (Fig. 5*C*). Cotransfection of a kinase inactive IKK1 plasmid did also not affect the elevated basal NF-κB reporter activity in LAMA84 cells. In contrast, this activity was substantially reduced by cotransfection of a kinase-inactive IKK2 plasmid, almost to the same degree as seen with cotransfection of an IκBαAA plasmid that expresses the IκBα substrate mutated at $Ser^{32/36}$ and completely blocks NF-κB activation (Fig. 5*C*). Thus, activation of NF-κB in LAMA84 cells is mainly due to IKK2 activity. Cotransfection of the PKD2-Y438D construct increased NF-κB reporter activity, whereas cotransfection of the PKD2-Y438F mutant significantly reduced the basal NF-κB reporter activity compared with both wild-type PKD2 and the PKD2-Y438D mutant (Fig. 5*C*). Kinase-inactive PKD2-D695A as well as constitutively active PKD2-S706/710E had no significant effect on the elevated basal NF-κB reporter activity. Thus, Bcr-Abl–induced NF-κB activity

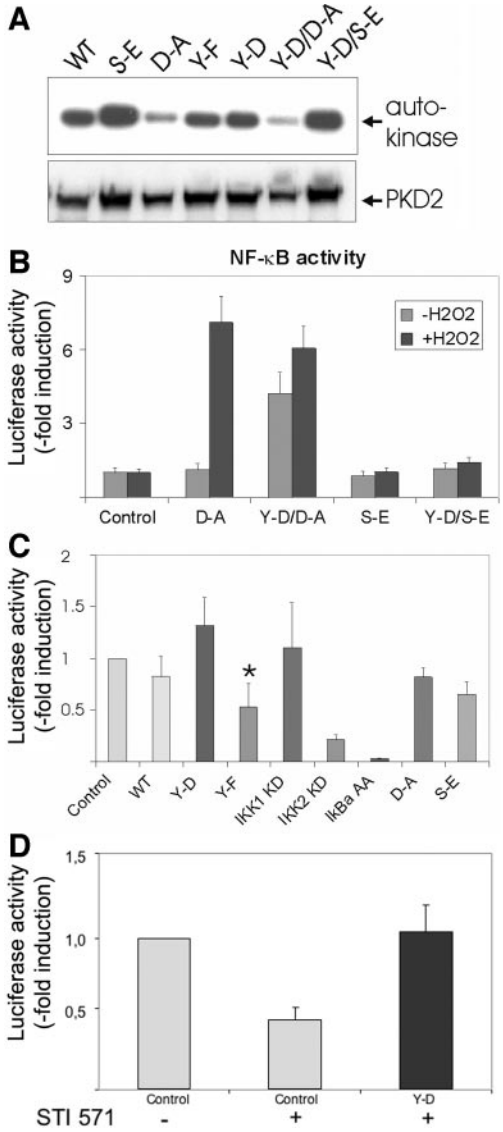

Fig. 5. *A*, autokinase activity of various PKD2 mutants. *Top panel*, to determine the autokinase activity of the various mutants, HEK293 cells were transfected with the respective FLAG-tagged expression plasmids (wild-type PKD2, *WT*; PKD2-S706/710E, *S-E*; PKD2-D695A, *D-A*; PKD2Y438F, *Y-F*; PKD2-Y438D, *Y-D*; PKD2-Y438D/D695A, *Y-D/D-A*; PKD2-Y438D/S706/710E, *Y-D/S-E*). Forty eight hours after transfection, cells were lysed, PKD2 was immunoprecipitated using an anti-FLAG antibody, and kinase activity was determined as described (autokinase). *Bottom panel*, aliquots of the cell lysates were analyzed by SDS-PAGE followed by anti-FLAG Western blotting (*arrow*, *PKD2*). *B*. PKD2-induced activation of NF-κB is independent of its kinase activity. HEK293 cells were cotransfected with NF-κB firefly luciferase, control *Renilla* luciferase, and the respective PKD2 mutants (PKD2-D695A, *D-A*; PKD2-Y438D/D695A, *Y-D/D-A*; PKD2-S706/710E, *S-E*; PKD2-Y438D/S706/710E, *Y-D/S-E*) as indicated for 16 hours and subsequently incubated with 250 nmol/L H_2O_2 (+*H2O2*) or solvent (−*H2O2*) for another 24 hours. NF-κB activity was determined using a NF-κB firefly luciferase reporter assay. *C*. NF-κB activity in LAMA84 cells can be regulated by PKD2. LAMA84 cells were cotransfected with NF-κB firefly luciferase and *Renilla* luciferase reporter plasmids and the various pcDNA3-FLAG-PKD2 expression plasmids as indicated (wild-type PKD2, *WT*; PKD2-Y438D, *Y-D*; PKD2-Y438F, *Y-F*; PKD2-D695A, *D-A*; PKD2-S706/710E, *S-E*), kinase inactive IKK1 (*IKK1 KD*) or IKK2 (*IKK2 KD*), or mutated IκBα that cannot be be phosphorylated by IKK (*IkBa AA*), respectively. Twenty four hours after transfection, the activities of firefly and *Renilla* luciferase were determined as described in Materials and Methods. *D*. Inhibition of constitutive NF-κB activation in LAMA84 cells by STI-571 is reversed by tyrosine-phosphorylated PKD2. LAMA84 cells were cotransfected with the NF-κB firefly luciferase and *Renilla* luciferase expression plasmids and either empty pcDNA vector (*Control*) or the PKD2-Y438D plasmid (*Y-D*) as indicated. After electroporation, cells were incubated with 250 nmol/L STI-571 for 48 hours (+) or received an equivalent amount of solvent (−). Subsequently, activities of firefly and *Renilla* luciferase were determined as described in Materials and Methods.

in LAMA84 cells is critically dependent on PKD2 phosphorylated at Tyr^{438}. This conclusion is further supported by the fact that the PKD2-Y438D mutant completely prevented the inhibition of basal NF-κB reporter activity by STI-571 in LAMA84 cells (Fig. 5*D*).

DISCUSSION

The PKD family of serine threonine kinases comprises three members that exhibit an overall similarity of >60% (7, 9, 13). Their biological role could differ due to the induction of distinct signaling pathways and also due to cell type-specific expression. We demonstrate that PKD2 is the major isoform of the PKD family expressed in Bcr-Abl^+ human myeloid leukemia cells. PKD2 was found to be phosphorylated in the PH domain at Tyr^{438} by Bcr-Abl in these cells, a site corresponding to Tyr^{463} in PKD1. PKD1 is phosphorylated at Tyr^{463} by Abl in response to oxidative stress (10), which leads to activation of the kinase (11) and subsequent activation of NF-κB. Therefore, it has been proposed that tyrosine-phosphorylated, catalytically active PKD1 could regulate one of the putative upstream kinases involved in IKK complex activation (10). These data link PKD2 to constitutive NF-κB activation in LAMA84 cells. A wide variety of malignancies including leukemias and epithelial tumors exhibit elevated basal activity of NF-κB. The activation mechanisms remain largely inconclusive. Our data indicate that the Bcr-Abl-PKD2-IKK pathway is a major component in LAMA84 cells. However, the composition of the nuclear NF-κB analyzed by supershift assays suggests a mixture of activation pathways. In our experiments, we investigated the endogenous IKK complex, indicating the involvement of the classical IKKβ-IκB pathway. However, we cannot rule out involvement of the alternative IKKα-p100 activation, which has been implicated in aberrant growth regulation and/or suppression of apoptosis in lymphocytes (14).

Our data show that, similar to PKD1, PKD2 mediates NF-κB activation by oxidative stress. This activation requires phosphorylation of the kinase at Tyr^{438}. However, tyrosine phosphorylation at this site does not lead to a marked increase in PKD2 kinase activity. Furthermore, a catalytically inactive but tyrosine-phosphorylated PKD2 mutant also stimulates NF-κB reporter activity, whereas a constitutively active PKD2 mutant fails to do so, even in the presence of an additional mutation that mimicks phosphorylation of PKD2 at Tyr^{438}. Thus, in marked contrast to PKD1, kinase activity and tyrosine phosphorylation of PKD2 represent two distinct and potentially even antagonistic signaling properties of the protein. Tyrosine-phosphorylated PKD2 is therefore more likely to serve as a docking site for interacting proteins that directly control IKKs because PKD2 kinase activity is not required for its effect on NF-κB activity. This finding is relevant for Bcr-Abl^+ human myeloid leukemia cells in which PKD2 is tyrosine phosphorylated but not constitutively active. The data also demonstrate that there are similarities but also striking differences regarding the regulatory function of the PH domain in various members of the PKD family.

In conclusion, PKD2 phosphorylated at Tyr^{438} is a novel mediator of NF-κB activation by Bcr-Abl in human myeloid leukemia cells. Because NF-κB activation plays a role in Bcr-Abl–induced cell transformation (2), targeting tyrosine phosphorylation of PKD2 in the PH domain—and not its kinase activity—may represent a novel therapeutic approach for Bcr-Abl^+ myeloid leukemia.

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