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Loss of Cell–Cell Contacts Induces NF-κB Via RhoA-Mediated Activation of Protein Kinase D1

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ABSTRACT

Cell–cell contacts mediated by cadherins are known to inhibit the small Rho-GTPase RhoA. We here show that in epithelial cells the disruption of these cell–cell contacts as mediated by a calcium switch leads to actin re-organization and the activation of RhoA. We identified the serine/threonine kinase protein kinase D1 (PKD1) as a downstream target for RhoA in this pathway. After disruption of cell–cell contacts, PKD1 relayed RhoA activation to the induction of the transcription factor NF-κB. We found that a signaling complex composed of the kinases ROCK, novel protein kinase C (nPKC), and Src family kinases (SFKs) is upstream of PKD1 and crucial for RhoA-mediated NF-κB activation. In conclusion, our data suggest a previously undescribed signaling pathway of how RhoA is activated by loss of cell–cell adhesions and by which it mediates the activation of NF-κB. We propose that this pathway is of relevance for epithelial tumor cell biology, where loss of cell–cell contacts has been implicated in regulating cell survival and motility. J. Cell. Biochem. 106: 714–728, 2009.

KEY WORDS: Rho; NF-κB; PKD; Src

Cell junctions are crucial for maintaining the integrity of epithelial tissues and are often disrupted as tumors progress to high-grade advanced disease [Hiscox and Jiang, 1997]. Cell–cell contacts of epithelial cells are mainly mediated by calcium-dependent adherens junctions. Adherens junctions are necessary to maintain epithelial structures and consist of membrane-spanning cadherin transmembrane receptors. Cadherins maintain the connection to the actin cytoskeleton, through interactions of their cytoplasmatic domain with catenins [Wheelock and Johnson, 2003a]. Coordinated regulation of the actin cytoskeleton by different Rho-GTPases is required for both the formation and disassembly of adherens junctions [Fukata and Kaibuchi, 2001]. RhoA activity is required for a very early step in junction formation leading to cadherin clustering at sites of cell–cell contacts [Braga et al., 1997; Takaishi et al., 1997]. However, during the remaining process of cell–cell contact formation, RhoA activity is decreased, whereby Rac and Cdc42 are increasingly active [Anastasiadis et al., 2000; Noren et al., 2000]. This is probably regulated by p120, which after binding to the cadherin/catenin complexes inhibits RhoA and activates Rac and Cdc42 [reviewed in Anastasiadis, 2007].

The assembly and maintenance of adherens junctions require calcium. Thus, EDTA-mediated calcium switch experiments leading to the disruption of cell–cell contacts are critical tools to induce and investigate signaling processes involving adherens junctions. For example, calcium switch experiments have revealed that disruption of cell–cell contacts promotes the re-organization of actin structures [Walsh et al., 2001]. The disruption and eventually loss of cell–cell contacts in tumor cells can be caused by downregulation of the expression of E-cadherin or catenin family members and leads to actin re-organization through Rho-GTPases [Wheelock and Johnson, 2003a,b]. This can contribute to enhanced tumor cell migration and proliferation, and plays an important role in processes such as epithelial-to-mesenchymal transition (EMT), where cancer cells loose E-cadherin expression to develop a more mesenchymal phenotype [Kang and Massague, 2004].

The Rho-GTPase family includes approximately 20 proteins of which the prototypic members RhoA, Rac1, and Cdc42 have been

Abbreviations used: Dia1, diaphanous-related formin 1; EMT, epithelial-to-mesenchymal transition; NF-κB, nuclear factor-κB; PKC, protein kinase C; PKD, protein kinase D; SFK, Src family kinase; ROCK, Rho kinase.

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best characterized [Sahai and Marshall, 2002a]. These proteins have been implicated in almost all cellular functions that underlie cancer progression including cell cycle, gene expression, matrix remodeling, focal adhesion assembly and disassembly [Burridge and Wennerberg, 2004]. "Globally" active RhoA inhibits the invasiveness of epithelial breast cancer cells [Wang et al., 2007]. However, local and temporal activation of RhoA is also required for lamellipodia extension and chemotaxis at the leading edge of migrating breast tumor cells [Arthur and Burridge, 2001; Wang et al., 2007]. An additional layer of complexity is provided by the fact that the different Rho isoenzymes are functionally distinct [Hall, 1998; Wang et al., 2003]. For example, although sharing high sequence homology, in invasive breast cancer cells, RhoA and RhoC differently contribute to tumorigenicity with RhoC enhancing and RhoA decreasing cell invasion [Simpson et al., 2004].

Two downstream effectors of RhoA, Rho-kinase (ROCK), and diaphanous-related formin (Dia1) regulate actin organization and may have opposing effects on adherens junction formation and maintenance [Sahai and Marshall, 2002b]. RhoA recently also has been implicated in the activation of protein kinase D1 (PKD1), a kinase that binds to F-actin and is involved in actin re-organization [Yuan et al., 2001; Li et al., 2004; Song et al., 2006; Eiseler et al., 2007]. PKD1 co-immunoprecipitates and co-localizes with E-cadherin at cell junctions in prostate cancer cells. Additionally, PKD1 has been shown to upregulate E-cadherin expression, and to promote cellular aggregation and decreased cellular motility by phosphorylating E-cadherin [Jaggi et al., 2005]. Further, PKD activated by RhoA protects against oxidative stress-induced intestinal epithelial cell injury via its activation through a ROCK and protein kinase Cδ (PKCδ)-mediated signaling pathway [Song et al., 2006].

PKD enzymes belong to the calcium/calmodulin-dependent kinase (CaM-K) family [Manning et al., 2002; Van Lint et al., 2002] and comprise a subset of three members, PKD1, PKD2, and PKD3, which have some overlapping, but also independent functions within cells [Yeaman et al., 2004; Auer et al., 2005; Chen et al., 2005; Hausser et al., 2005; Sanchez-Ruiloba et al., 2006; Wang, 2006; Doppler and Storz, 2007]. Both PKD1 and PKD2 have been shown to activate the inducible transcription factor NF-κB [Storz and Toker, 2003; Mihailovic et al., 2004], which is a sensor for actin re-organization [Montaner et al., 1998; Benitah et al., 2003; Anwar et al., 2004; Yan et al., 2005]. For example, the disruption of the actin cytoskeleton with Cytochalasin D leads to the RhoA-dependent activation of NF-κB in many tumor cell lines [Ren et al., 1999; Nemeth et al., 2004]. PKD1/2-mediated induction of NF-κB is dependent on a tyrosine phosphorylation-mediated activation mechanism. We and others have shown that tyrosine phosphorylation of PKD1, mediated either directly by Src family kinases (SFKs) or in a SFK-dependent manner [Waldron and Rozengurt, 2000; Storz and Toker, 2003; Storz et al., 2003], regulate PKD1's ability to activate NF-κB. Tyrosine phosphorylation further results in subsequent activation-loop phosphorylation of PKD1 by novel PKC (nPKC: PKCε, PKCδ, PKCη, PKCθ) enzymes [Waldron and Rozengurt, 2000; Storz et al., 2003, 2004a,b; Doppler and Storz, 2007]. The activation-loop phosphorylation of PKD directly correlates with its activity [for a review on PKD activation mechanisms: Wang, 2006]. So far, oxidative stress in epithelial cells, expression of BCR-Abl in hematopoietic tumor cells, and lipid raft disruption in neuronal cells, but none of the other known regulators of PKD1 signaling, have been shown to induce tyrosine phosphorylation of this kinase [Storz and Toker, 2003; Storz et al., 2003; Cabrera-Poch et al., 2004; Mihailovic et al., 2004].

The exact mechanisms of how RhoA activates NF-κB were not identified so far, nor were upstream activators of RhoA identified that lead to this signaling. Since NF-κB is known to function as a sensor of actin re-organization, and since altered cell–cell adhesion had been described to regulate actin remodeling and RhoA activity, we here investigated if a PKD1-regulated signaling mechanism is involved in RhoA-mediated regulation of NF-κB.

RESULTS

LOSS OF CELL–CELL CONTACTS BY Ca^{2+} DEPLETION INDUCES ACTIN AND PKD1 RE-ORGANIZATION

Depletion of cell culture medium from calcium (Ca^{2+}) is known to disrupt cell–cell contacts of epithelial cells by dismissing cadherin interactions [Walsh et al., 2001]. Consequently, Ca^{2+} depletion, mediated by a 15-min treatment with EDTA resulted in a loss of cell–cell contacts of epithelial HeLa cells (Fig. 1A). Notably, no changes in cell–extracellular matrix adhesion were detected in a time range up to 1 h (data not shown), suggesting that integrin–extracellular matrix connections stayed intact after Ca^{2+} depletion.

Cadherins not only mediate cell–cell contacts, but also link the cell surface to the actin cytoskeleton, and loss of cadherin–cadherin interactions leads to actin remodeling within cells [Wheelock and Johnson, 2003a]. In HeLa cells, the disruption of these interactions by an EDTA-mediated calcium switch induced the re-organization and re-localization of actin (Fig. 1B, left pictures, red). In cells with functional cell–cell contacts, F-actin was localized at lamellipoda in membrane ruffles and protrusions (Fig. 1B, two upper left panels). An increased redistribution of F-actin was noticed after loss of cell–cell contacts induced by the Ca^{2+} depletion (Fig. 1B, two lower left panels). This redistribution of actin correlated with a re-localization of the serine/threonine kinase PKD1 from lamellipodia, where it was co-localized with actin in untreated cells (Fig. 1B, two upper right panels, overlay), to the sites of actin re-organization after disruption of cell–cell contacts (Fig. 1B, two lower right panels, overlay). Notably, a significant amount of PKD1 was localized in the nucleus. Further, tyrosine phosphorylation of PKD1 at a tyrosine residue (Y95) critical for its activation by other stimuli [Doppler and Storz, 2007] occurred after a Ca^{2+} switch in HeLa and MCF-7 cells (Fig. 1C), indicating that PKD1 is active at the sites of actin re-organization, but not the nucleus. We therefore analyzed if this re-localization of PKD1 leads to its activation.

Ca^{2+} DEPLETION INDUCES PKD1 ACTIVATION BY A TYROSINE PHOSPHORYLATION-DEPENDENT MECHANISM

Since the translocation of PKD can lead to its activation [Mullin et al., 2006], we investigated if loss of cell–cell contacts, induced by Ca^{2+} depletion, can activate PKD1. We analyzed both PKD1 auto- and substrate phosphorylation in response to a calcium switch

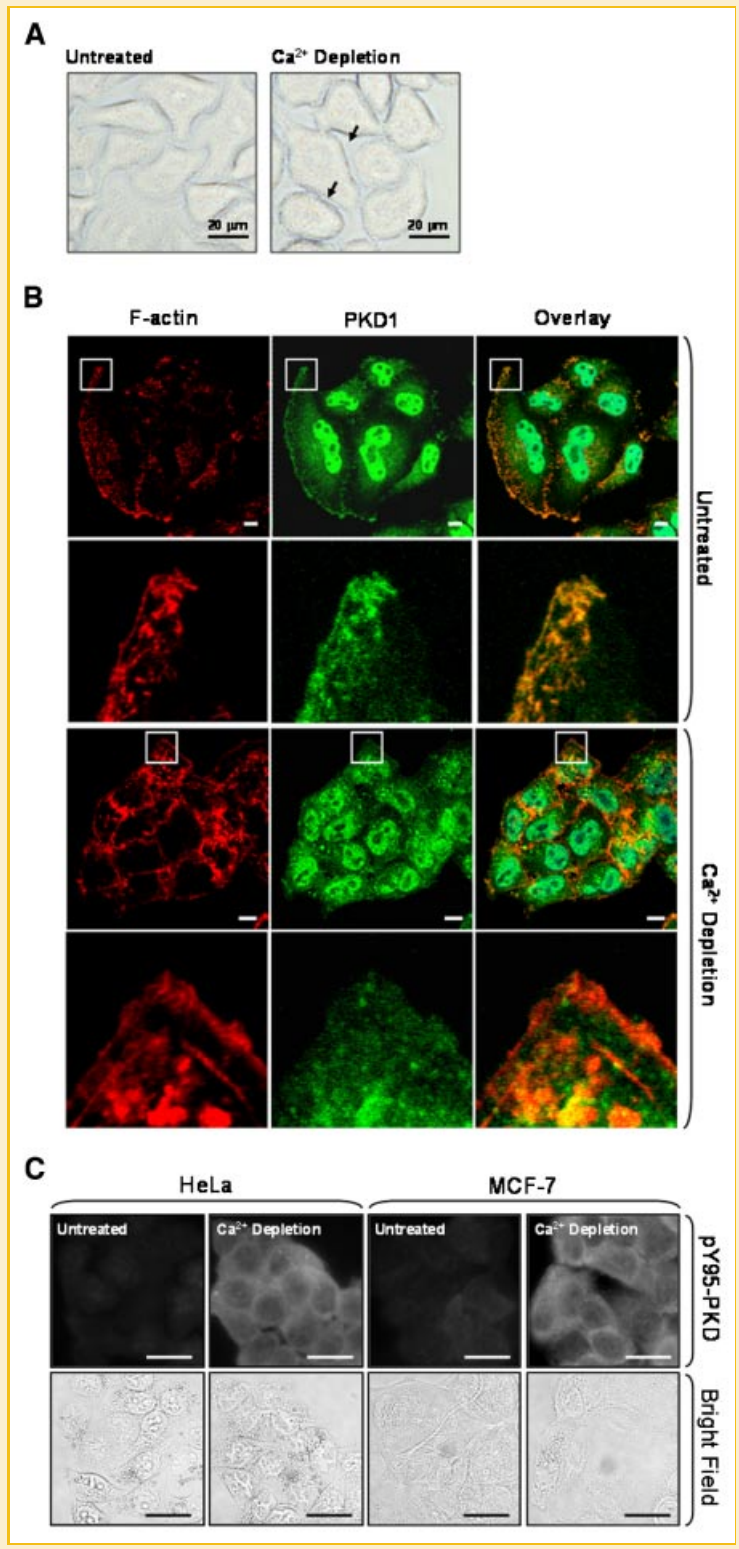
A
Untreated
Ca2+ Depletion
20 μm
20 μm
B
F-actin
PKD1
Overlay
Untreated
Ca2+ Depletion
C
HeLa
MCF-7
Untreated
Ca2+ Depletion
Untreated
Ca2+ Depletion
pY95-PKD
Bright Field

Fig. 1.

(Fig. 2A,B). Stimulation of cells with H_2O_2, a potent activator of PKD1 [Storz and Toker, 2003; Waldron et al., 2004], served as a positive control (Fig. 2A). PKD1-mediated substrate phosphorylation was measured using a synthetic peptide as a substrate (Fig. 2B). Depletion of cell culture media from calcium resulted in PKD activation with a peak at 10 min as measured by auto- and substrate phosphorylation in vitro kinase assays after immunoprecipitation of endogenous PKD (Fig. 2A,B).

Both tyrosine phosphorylations as well as the phosphorylation at two critical serine residues in the activation loop of PKD1 have been directly linked to the activation of this kinase [Storz et al., 2004a]. PKD1 tyrosine phosphorylations are mediated by SFK [Waldron and Rozengurt, 2000; Storz and Toker, 2003]. So far, this activation mechanism has been described for oxidative stress-mediated PKD1/2 activation and after lipid raft disruption in neuronal cells [Storz et al., 2003, 2004a,b; Cabrera-Poch et al., 2004]. Other PKD1 activation mechanisms are most likely independent of tyrosine phosphorylation events [Storz, 2007]. On the other hand, all known activation mechanisms eventually lead to activation-loop phosphorylations in the kinase domain, which has been shown to correlate with PKD1 activity [Waldron et al., 1999; Waldron and Rozengurt, 2003]. For human PKD1 activation loop, phosphorylation at S738 and S742 is mediated by nPKC enzymes [Waldron and Rozengurt, 2003]. Therefore, we next analyzed the PKD1 phosphorylation status in response to a Ca^{2+} switch-mediated loss of cell–cell contacts. We found that after Ca^{2+} depletion, PKD1 is phosphorylated at tyrosine residue Y95 and at the activation-loop serines S738 and S742 in HeLa and MCF-7 cells (Fig. 2C,D). This indicates the involvement of the SFK and nPKC enzymes in Ca^{2+} depletion-mediated activation of PKD1. To determine if the Ca^{2+} switch indeed mimics the disruption of E-cadherin–E-cadherin complexes, we alternatively used an E-cadherin blocking antibody. The blockage of E-cadherin–E-cadherin connections also led to PKD1 phosphorylation at Y95 and the activation-loop serines (Fig. 3E).

Ca^{2+} DEPLETION LEADS TO ACTIVATION OF RhoA AND RhoA-DEPENDENT Y95 AND ACTIVATION-LOOP PHOSPHORYLATION OF PKD1

Both regulation of cell–cell contacts as well as actin re-organization are tightly linked to the Rho-GTPase family [Takaishi et al., 1997; Fukata and Kaibuchi, 2001; Wheelock and Johnson, 2003a,b]. For example, the loss of E-cadherin interactions has been shown to activate RhoA. We therefore tested if Ca^{2+} depletion leads to increased RhoA activation in HeLa cells. The disruption of cell–cell contacts significantly increased active GTP-bound RhoA as measured in pull-down assays with GST-Rhotekin (Fig. 3A). We then expressed constitutive-active alleles of the Rho-GTPases Cdc42, Rac1, and RhoA and analyzed their contribution to PKD1 activation. Only the expression of a constitutively active RhoA significantly induced PKD1 activation-loop phosphorylation (Fig. 3B). Of note, the expression of other Rho isoenzymes such as RhoC also had no effect on PKD1 activity (Supplemental Fig. 2). We then used a reciprocal approach to determine if RhoA is responsible for the activation of PKD1 after Ca^{2+} depletion. Therefore, we expressed either dominant-negative RhoA or RhoB as control, induced loss of cell–cell adhesion by Ca^{2+} depletion, and measured PKD1 activation-loop phosphorylation by using phospho-specific antibodies (Fig. 3C). The expression of dominant-negative RhoA blocked the activation of PKD1 by Ca^{2+} depletion, indicating that the signaling events that lead to PKD1 activation are mediated by the small GTPase RhoA. Similar to the data obtained after calcium depletion (Fig. 2C), expression of constitutively active RhoA induced both PKD1 phosphorylation at critical tyrosine residues and activation-loop serines (Fig. 3D). This implicates that loss of cell–cell contacts activates PKD1 via RhoA.

RhoA ACTIVATES PKD1 VIA ROCK, SFK, AND nPKC

We next investigated how RhoA activates PKD1 in response to calcium depletion. Key events for PKD1 activation are phosphorylations at the activation loop, which directly correlate with PKD1 activity [Waldron et al., 1999; Waldron and Rozengurt, 2003]. It was further shown that PKD1 activation can be dependent or independent of SFK-mediated tyrosine phosphorylations [Storz and Toker, 2003; Storz et al., 2003, 2004b]. A key tyrosine phosphorylation site in PKD1 is tyrosine residue 95 [Doppler and Storz, 2007]. The occurrence of tyrosine phosphorylations in RhoA signaling suggests a direct involvement of SFKs in the RhoA-mediated PKD1 activation process. By comparing cells that were pretreated with the SFK inhibitor PP2 [Storz and Toker, 2003] and transfected with active RhoA, we found that PP2 inhibited RhoA-mediated phosphorylation of PKD1 at Y95 (Fig. 4A).

To test if Src is involved in Y95 phosphorylation of PKD1 in response to active RhoA, we compared control cells with cells which expressed wild-type and dominant-negative Src and measured PKD1 activity in response to active RhoA (Fig. 4B). The expression of wild-type Src in absence of RhoA as an activating stimulus had no effect on PKD1 activity (Fig. 4B, lane 2). However, wild-type Src significantly increased RhoA-mediated tyrosine phosphorylation (measured with anti-pY95) and this was blocked when a dominant-negative Src was expressed (Fig. 4B, lanes 5 and 6). This indicates that Src is downstream of RhoA in this activation pathway.

Fig. 1. A calcium switch induces loss of HeLa cell–cell contacts, actin and PKD1 re-organization, and PKD1 activation. A: 0.5×10^6 HeLa cells were treated with 0.5 M EDTA solution for 15 min to induce a calcium switch. Cells were photographed in bright field using a 40× lens. The scale bar represents 20 μm. The arrows indicate example areas with loss of cell–cell contacts. B: 6×10^4 HeLa cells were seeded on coverslips. Loss of HeLa cell–cell contacts was induced by Ca^{2+} depletion (EDTA, 0.5 M, 15 min). Cells were fixed and F-actin and PKD1 were stained by immunofluorescence. Localization of both proteins was determined by confocal microscopy. Shown are staining for F-actin (Rhodamine–Phalloidin) and PKD1 (anti-PKD1) as well as a composite. The scale bar represents 10 μm. The areas marked by a square are enlarged to show co-localization of PKD1 with F-actin in untreated cells, and loss of this interaction in EDTA-treated cells. C: 5×10^4 HeLa or MCF-7 cells were seeded on coverslips, treated with 0.5 M EDTA solution for 15 min to induce a calcium switch, fixed and tyrosine95-phosphorylated PKD was stained by immunofluorescence using an anti-pY95 antibody. Cells were analyzed by microscopy. All results are typical of multiple independent experiments.

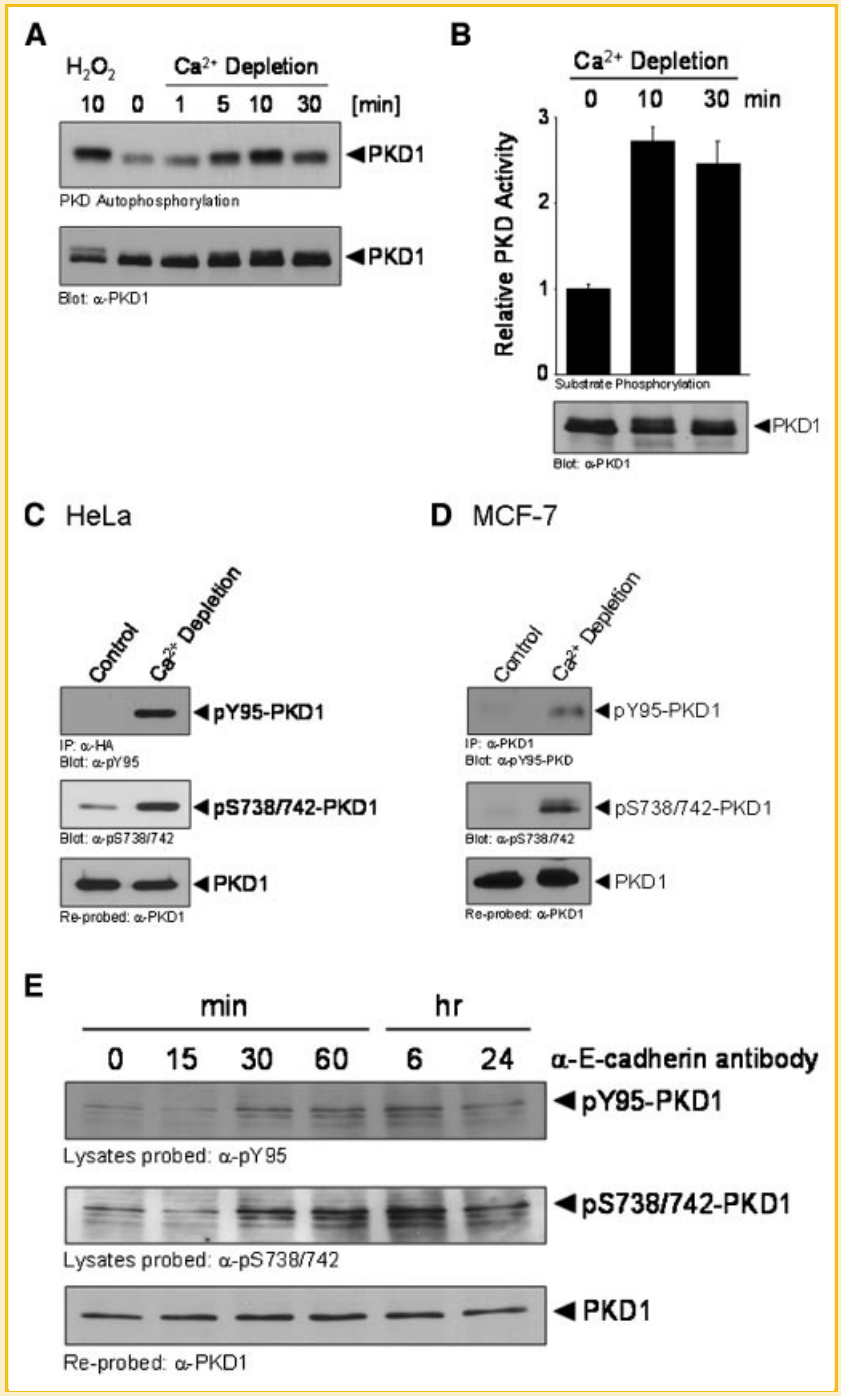

Fig. 2. Ca^{2+} depletion induces PKD1 activation by a tyrosine phosphorylation-dependent mechanism. A,B: 2×10^6 HeLa cells were treated with 0.5 M EDTA solution in a time kinetic to induce the loss of cell–cell contacts by a calcium switch, or treated for 10 min with 10 mM H_2O_2 as a positive control. PKD1 was immunoprecipitated and kinase assays were performed to measure PKD1 autophosphorylation (A) or substrate phosphorylation (B). Equal levels of immunoprecipitated PKD1 were controlled in immunoblot analysis using a PKD1-specific antibody. C: 10^6 HeLa cells transfected with HA-tagged PKD1 were subjected to Ca^{2+} depletion (EDTA, 0.5 M, 15 min) or left untreated. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations at tyrosine residue Y95 (anti-pY95) and serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. D: 3×10^6 MCF-7 cells were subjected to Ca^{2+} depletion (EDTA, 0.5 M, 15 min) or left untreated. Endogenous PKD1 was immunoprecipitated (anti-PKD1). Samples were further processed as in (C). E: 10^6 HeLa cells were transfected with HA-tagged PKD1 and then incubated with 4 μg/ml of an E-cadherin blocking antibody (mouse anti-human E-cadherin antibody) for indicated times. Cells were lysed and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations at tyrosine residue Y95 (anti-pY95), serine residues S738 and S742 (anti-pS738/742), or PKD1 (anti-PKD1). All results are typical of three independent experiments.

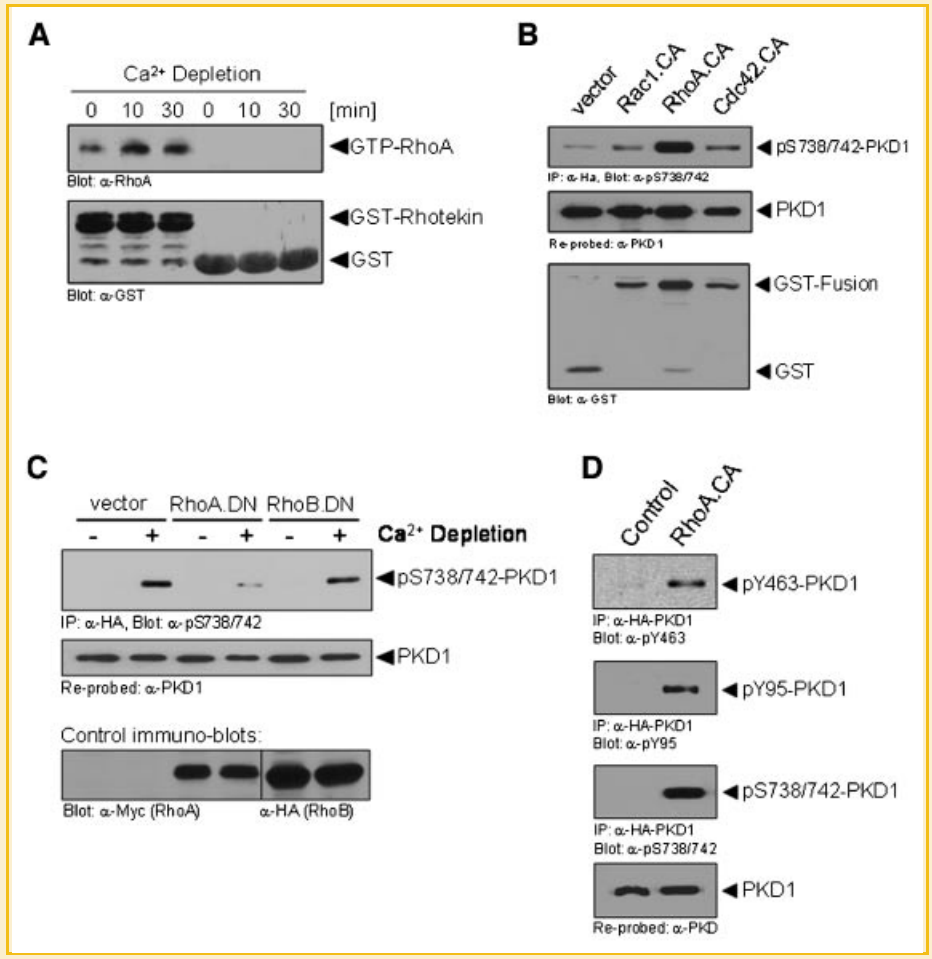

Fig. 3. Ca^{2+} depletion leads to activation of RhoA and RhoA-dependent phosphorylation of PKD1. A: 3×10^6 HeLa cells were treated with 0.5 M EDTA solution in a time kinetic to induce a calcium switch. Lysates were incubated with GST-Rhotekin to pull down GTP-bound Rho protein or with GST alone (specificity control). Samples were separated in SDS–PAGE, transferred to nitrocellulose, and analyzed for pull-down Rho by immunoblot analysis (anti-RhoA). Equal use of GST fusion and GST protein for pull down was verified by immunoblot analysis (anti-GST). B: 10^6 HeLa cells were transfected with a HA-tagged PKD1 and constitutively active GST-tagged expression constructs for Rac1, RhoA, Cdc42, or pEBG as a vector control. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations at activation-loop serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. GST-Rho-GTPase fusion protein and GST control expression was controlled by immunoblot analysis using an anti-GST antibody. C: 10^6 HeLa cells were transfected with a HA-tagged PKD1 and dominant-negative RhoA (MYC-tagged) or RhoB (HA-tagged) or vector control. Cells were then subjected to Ca^{2+} depletion (EDTA, 0.5 M, 15 min) or left untreated. PKD1 was immunoprecipitated (anti-HA), samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations at activation-loop serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. Expression of dominant-negative RhoA and RhoB was controlled in immunoblot analysis using anti-MYC and anti-HA antibodies. D: 10^6 HeLa cells were transfected with a HA-tagged PKD1 and vectors to express constitutively active RhoA or controls. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations at tyrosine residues Y463 (anti-Y463), Y95 (anti-pY95), and serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. All results are typical of three independent experiments.

We have previously shown for oxidative stress signaling that tyrosine phosphorylation of PKD1 by Src is prerequisite for its phosphorylation at the activation-loop serines [Doppler and Storz, 2007]. Activation-loop phosphorylation of PKD1 is mediated by nPKC enzymes. We next determined in a subset of experiments which of the four nPKC isoforms (δ, ε, η, θ) mediate PKD1 activation in response to activation of RhoA in HeLa cells. Therefore, we first determined the subset of nPKC isoforms expressed in HeLa cells. Our results show that HeLa cells only express PKCδ and PKCε (Fig. 5A). We then knocked down PKCδ or PKCε to determine their role in RhoA-mediated activation of PKD1. We found that the knockdown of PKCδ with specific siRNA had no effect on RhoA-mediated activation of PKD1 (Fig. 5B), but the knockdown of PKCε with specific siRNA completely blocked RhoA-mediated activation of PKD1 (Fig. 5C).

We next determined if PKD1 activating phosphorylations in response to a Ca^{2+} switch are dependent on the RhoA target kinase ROCK (Rho kinase). The treatment of HeLa cells with the ROCK

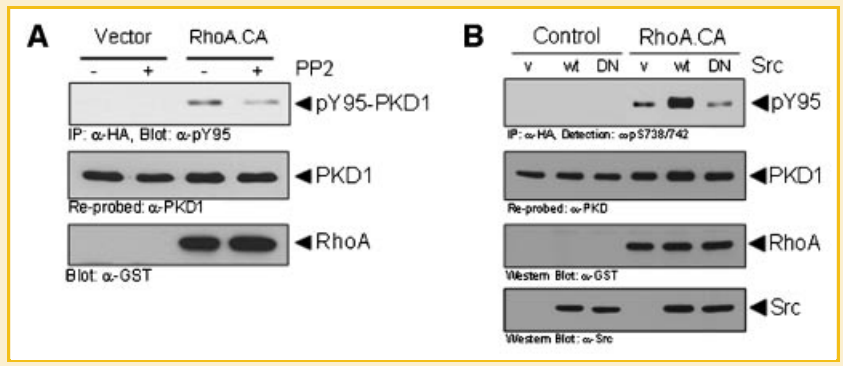

Fig. 4. Src family kinases contribute to RhoA-mediated activation of PKD1. A: 10^6 HeLa cells were transfected with vector control (vector) or GST-tagged constitutively active RhoA (RhoA-CA) and treated with the Src family kinase inhibitor PP2 (10 μM, 1 h) as indicated. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for SFK-mediated phosphorylations of PKD1 at Y95 using an anti-pY95 antibody. Blots were controlled for PKD1 and GST-RhoA expression. B: 10^6 HeLa cells were co-transfected with PKD1, vector control, or GST-tagged constitutively active RhoA (RhoA-CA) and vector control (V), wild-type Src (WT), or dominant-negative Src (DN) as indicated. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations of PKD1 at Y95 and the activation loop (anti-pS738/742). Blots were controlled for PKD1, GST-RhoA, and Src expression. Results are typical of three independent experiments.

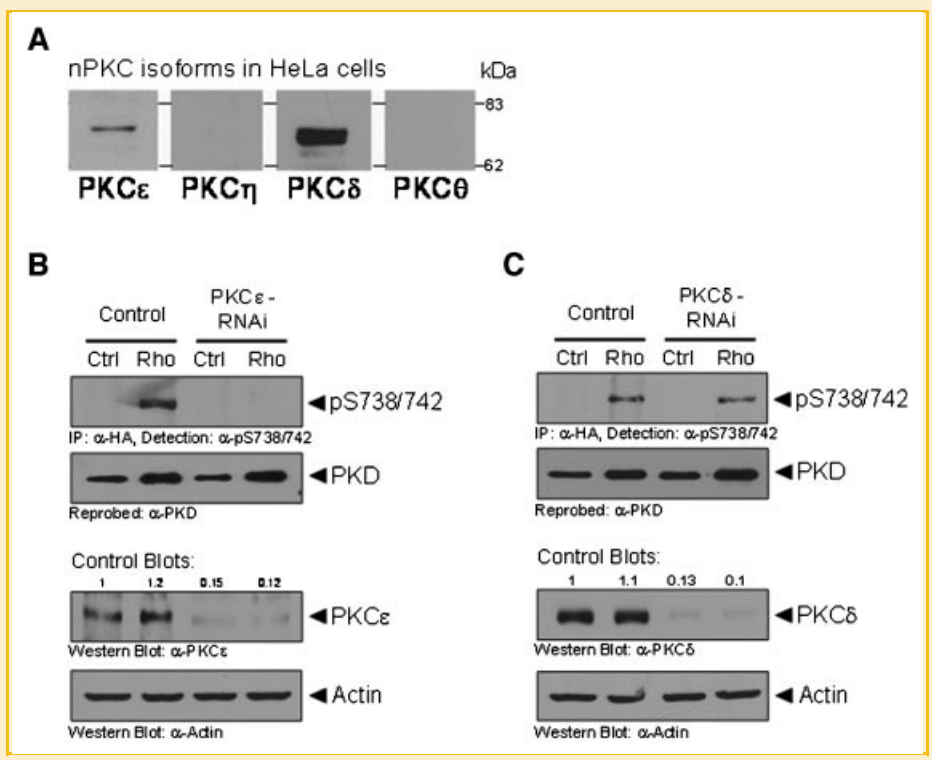

Fig. 5. RhoA-induced activation-loop phosphorylation of PKD1 is mediated by PKCε. A: Lysates of HeLa cells were analyzed by immunoblotting for nPKC expression using specific antibodies for the nPKC isoforms PKCε, PKCη, PKCδ, and PKCθ. B,C: 0.4×10^6 HeLa cells were transfected with control or PKCδ-RNAi or PKCε-RNAi expression constructs. After 24 h, cells were subjected to a second transfection with HA-tagged PKD1 and constitutively active RhoA or vector control. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylation at serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. Equal expression of RhoA and knockdown of PKC isoforms was determined by immunoblotting with anti-GST antibodies (RhoA) or anti-PKCδ and PKCε antibodies. All results are typical of three independent experiments.

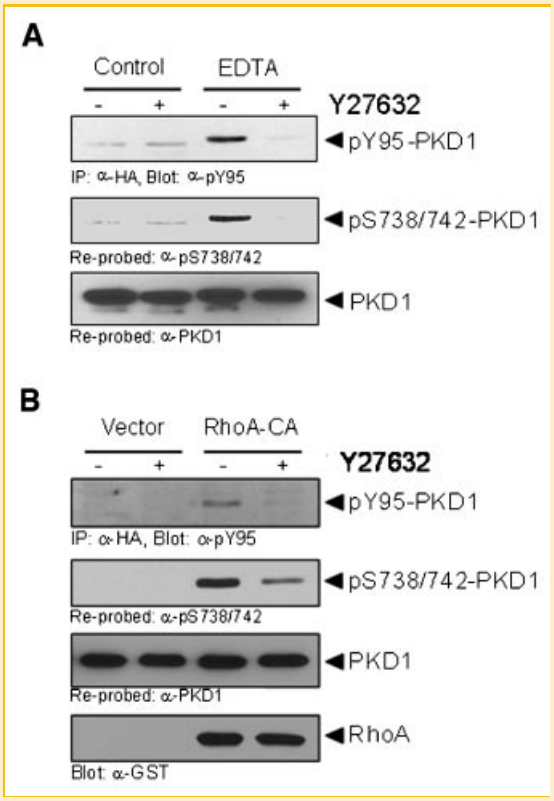

Fig. 6. Ca^{2+} depletion and RhoA activate PKD1 via ROCK. A: 10^6 HeLa cells were transfected with a HA-tagged PKD1. Cells were preincubated with the ROCK inhibitor Y27635 (10 μM, 1 h) as indicated. Cells were then subjected to Ca^{2+} depletion (EDTA, 0.5 M, 15 min) or left untreated. PKD1 was immunoprecipitated (anti-HA), samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylation of tyrosine residue Y95 (anti-pY95) and serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. B: 10^6 HeLa cells were transfected with vector control (vector) or constitutive-active RhoA (RhoA-CA) and treated with the ROCK inhibitor Y27635 (10 μM, 1 h) as indicated. PKD1 was immunoprecipitated (anti-HA) and samples were separated on SDS–PAGE, transferred to nitrocellulose, and analyzed by immunoblotting for phosphorylations at tyrosine residue Y95 (anti-pY95) and serine residues S738 and S742 (anti-pS738/742). Immunoprecipitates were re-probed with anti-PKD1 to show equal levels of immunoprecipitated PKD1. Expression of active RhoA was controlled in immunoblot analysis using anti-GST antibodies. Results are typical of three independent experiments.

inhibitor Y27632 [Maekawa et al., 1999] followed by Ca^{2+} depletion revealed that ROCK is involved in PKD1 activation (Fig. 6A). The inhibition of ROCK with Y27632 suggested both, that ROCK is upstream of PKD1 phosphorylation at Y95 (and thus upstream of the involved SFK) as well as the activation-loop serines (and thus upstream of the involved nPKC enzyme). We obtained similar results by expressing a constitutively active RhoA (Fig. 6B), implicating that ROCK indeed is downstream of RhoA in this signaling pathway, but also upstream of the kinases that mediate the phosphorylation of PKD1 at sites critical for its activation.

Taken together, our data implicate that PKD1 is activated by RhoA via a ROCK, SFK, and nPKC-dependent mechanism. They also implicate that ROCK regulates events in this signaling mechanism that are upstream of SFK and nPKC.

RhoA INDUCES NF-κB VIA PKD1

Both activation of RhoA as well as loss of cell–cell contacts have previously been described to activate the stress-responsive transcription factor NF-κB [Montaner et al., 1998; Benitah et al., 2003; Anwar et al., 2004; Yan et al., 2005; Liu et al., 2006]. The involved signaling pathways, however, have not been elucidated. Interestingly, tyrosine phosphorylation and activation of PKD1, mediated by the SFK Src and the nPKC enzyme PKCδ, have been shown to mediate the activation of NF-κB by inducers of oxidative stress [Storz and Toker, 2003; Storz et al., 2003, 2004a,b]. Therefore, we tested if RhoA, similar to oxidative stress, activates NF-κB through a PKD1-mediated signaling pathway.

Both mimicking PKD1 tyrosine phosphorylations (PKD1. Y463E) as well as mimicking activation-loop phosphorylations (PKD1.S738E/S742E) induced NF-κB in our cell system (Fig. 7A), whereby the PKD1.Y463E mutant mimics a tyrosine phosphorylation that causes SFK-mediated phosphorylation of Y95 [Doppler and Storz, 2007] and leads to PKD1 activity [Storz and Toker, 2003]. A PKD1.Y95E mutant could not be used since phosphorylation of this site does generate a protein-docking site and the mutation does not constitute a protein interaction site [in detail shown in Doppler and Storz, 2007]. We next analyzed if PKD1 can contribute to RhoA-mediated activation of NF-κB. The co-expression of wild-type PKD1 significantly enhanced RhoA-mediated activation of NF-κB (Fig. 7B). On the other hand, the expression of a kinase-dead PKD1 (Fig. 7C) or the knockdown of PKD expression (Fig. 7D) reduced RhoA-mediated NF-κB activation. Due to overlapping functions of PKD1 and PKD2, we knocked down both isoforms using specific RNAi [Storz et al., 2005b]. The reduction of PKD1/2 expression directly correlated with the decreased induction of NF-κB as compared to the control cells. These results indicate that PKD indeed contributes to RhoA-mediated activation of this transcription factor.

Ca^{2+} DEPLETION INDUCES NF-κB VIA A ROCK-PKD1 SIGNALING PATHWAY

Finally, we determined if the depletion of cell culture medium from calcium, which led to the activation of RhoA/ROCK signaling, induces NF-κB, and if this is mediated by PKD1. Therefore, we compared control cells to cells where we had knocked down PKD with specific RNAi. We found that NF-κB activation through Ca^{2+} depletion was blocked when PKD1 expression was downregulated with PKD-RNAi (Fig. 8A). In order to investigate if this EDTA-mediated NF-κB activation is due to RhoA/ROCK signaling, we compared cells treated with the ROCK inhibitor Y27632 to untreated cells and analyzed NF-κB activity. We found that loss of cell–cell adhesion as mediated by Ca^{2+} depletion led to NF-κB activation in a ROCK-dependent manner (Fig. 8B). This implicates that a calcium switch activates NF-κB through RhoA/ROCK and PKD1. In order to determine if PKD1, when activated by the above-described pathway, activates NF-κB via its canonical activation pathway, we expressed active RhoA, active PKD1, or induced the loss of cell–cell contacts with EDTA and analyzed the degradation of IκBα (Fig. 8C). Our data

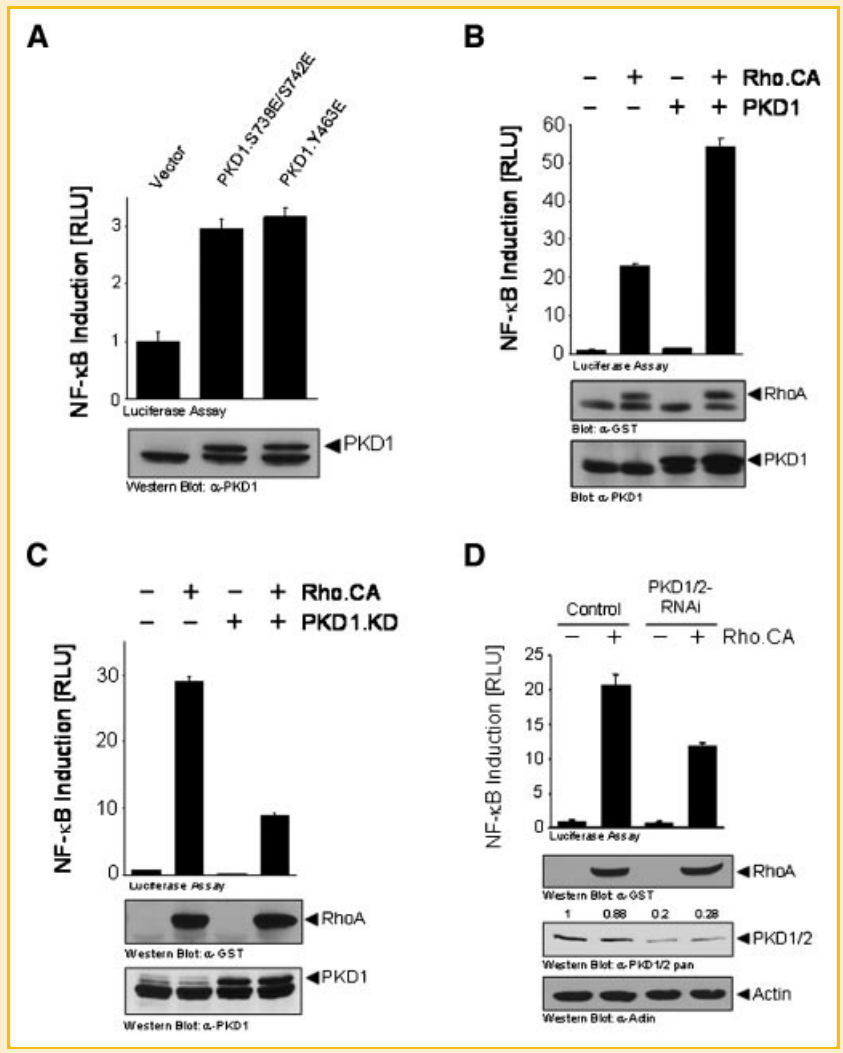

Fig. 7. RhoA induces NF-κB via PKD1. A: 0.4×10^6 HeLa cells were transfected with NF-κB and Renilla reporter genes and co-transfected with active PKD1 (PKD1.Y463E; PKD1.S738E/S742E) or vector control. Luciferase reporter gene assays were performed. NF-κB induction was normalized to Renilla. Protein expression was controlled by immunoblotting against indicated proteins. B: 0.4×10^6 HeLa cells were transfected with NF-κB and Renilla reporter genes and co-transfected with active RhoA and PKD1 or vector controls as indicated. Luciferase reporter gene assays were performed. NF-κB induction was normalized to Renilla. Protein expression was controlled by immunoblotting against indicated proteins. C: 0.4×10^6 HeLa cells were transfected with NF-κB and Renilla reporter genes and co-transfected with active RhoA and kinase-inactive PKD1 (PKD1.KD) or vector controls as indicated. Luciferase reporter gene assays were performed. NF-κB induction was normalized to Renilla. Protein expression was controlled by immunoblotting against indicated proteins. D: 0.2×10^6 HeLa cells were transfected with PKD-RNAi expression constructs or control. After 24 h, cells were subjected to a second transfection with NF-κB, Renilla reporter genes and active RhoA or vector control. Luciferase reporter gene assays were performed. NF-κB induction was normalized to Renilla. Protein expression was controlled by immunoblotting against indicated proteins. All results are typical of three independent experiments.

indicate that in response to the activation of the RhoA-PKD pathway, NF-κB is regulated via IκBα degradation and thus its canonical activation pathway.

Taken together, our data suggest a signaling pathway, which is summarized in Figure 9. We found that the loss of cell–cell contacts leads to activation of RhoA and induction of NF-κB. This RhoA-mediated NF-κB activation mechanism is controlled by the RhoA downstream kinase ROCK. We further show that RhoA and ROCK induce NF-κB through PKD1. PKD1 activation is mediated by phosphorylation through ROCK-regulated SFK and nPKC enzymes.

DISCUSSION

E-cadherin-mediated adherens junctions critically determine epithelial tissue organization and integrity, and the dysfunction of E-cadherin leads to the loss of cell–cell contacts which contributes to increased tumor cell invasion and metastasis. Cadherins actively modulate cell signaling through Rho-GTPases. For example, the initial processes involved in the formation of adherens junctions require RhoA activity. However, once these complexes are formed, RhoA is rapidly inactivated due to complex formation of E-cadherin with p120 [Anastasiadis, 2007]. RhoA's

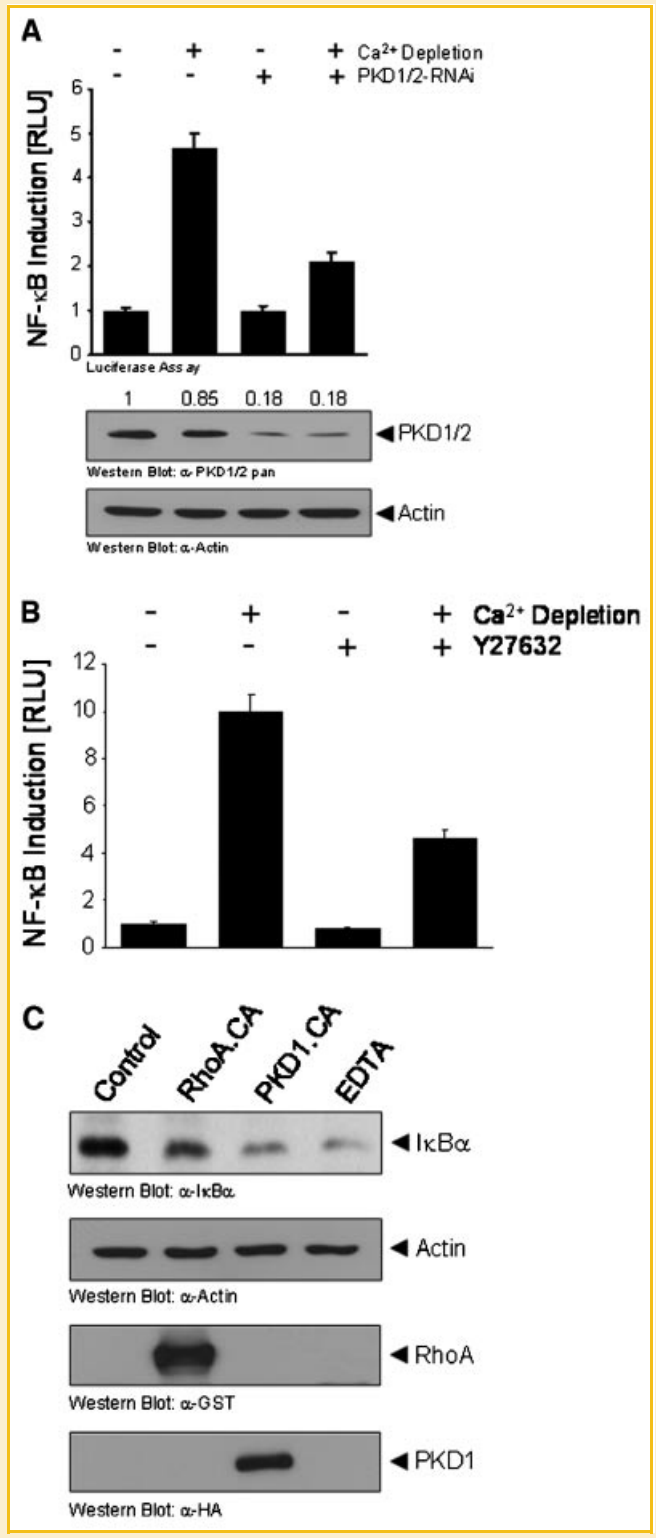

Fig. 8. Ca^{2+} depletion induces NF-κB via a RhoA-PKD1 signaling pathway. A: 0.2×10^6 HeLa cells were transfected with PKD-RNAi expression constructs or control. After 24 h, cells were subjected to a second transfection with NF-κB and Renilla reporter genes. Cells were treated with EDTA (0.4 M, 16 h) to induce a calcium switch as indicated. Luciferase reporter gene assays were performed. NF-κB induction was normalized to Renilla. PKD knockdown was controlled by immunoblotting against PKD1/2 (α-PKD-C20 from Santa Cruz) or actin as a loading control. B: 0.4×10^6 HeLa cells were transfected with NF-κB and Renilla reporter genes. Cells were treated with the ROCK inhibitor Y27632 (10 μM, 1 h) or left untreated. One hour after addition of Y27632, cells were treated with EDTA (0.4 M, 16 h) to induce a calcium switch as indicated. Luciferase reporter gene assays were performed. NF-κB induction was normalized to Renilla. C: 10^6 HeLa cells were transfected with vector control, constitutively active RhoA (RhoA-CA), constitutively active PKD1 (PKD1.Y463E) or were subjected to Ca^{2+} depletion (EDTA, 0.5 M, 15 min) as indicated. Samples were analyzed by immunoblotting for IκBα, actin, RhoA, and PKD1 expression. All results are typical of three independent experiments.

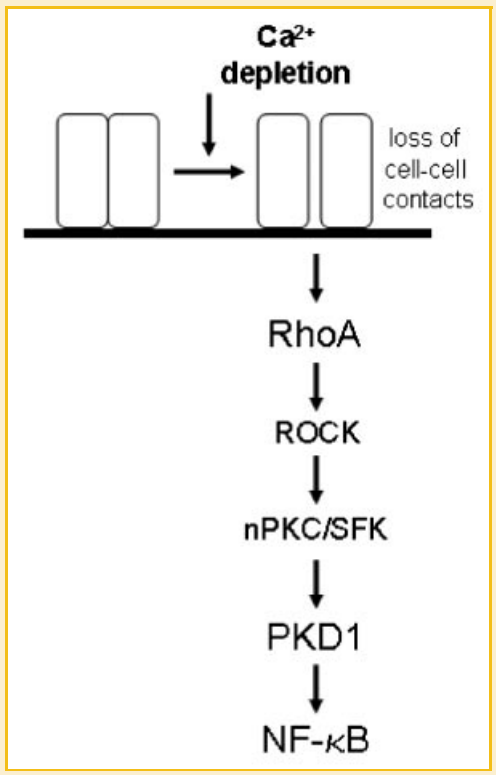

Fig. 9. Model of the proposed RhoA/PKD1/NF-κB signaling pathway. Disruption of cell–cell contacts as mediated by calcium depletion leads to the activation of RhoA. Active RhoA leads to the activation of the PKD1. PKD1 activation by RhoA is facilitated through activation of SFK (Src family kinases) and nPKC (novel PKC enzymes). The RhoA downstream kinase ROCK regulates both, phosphorylations mediated by SFK and nPKC. Active PKD1 relays the signal to the transcription factor NF-κB, which has been described to have important functions in cell adhesion and survival. The target genes for NF-κB in this signaling pathway are not defined, yet.

major function is in processes that control actin filament re-organization [Slater et al., 2001, 2003] and its regulation at adherens junctions may contribute to epithelial integrity. A critical step in cancer progression is the ability of tumor carcinoma cells to acquire altered adhesion and polarization characteristics through cytoskeletal rearrangements [Sahai and Marshall, 2002a]. We here describe that an EDTA-mediated calcium switch, which is known to disrupt E-cadherin-mediated cell–cell contacts of epithelial cells (Fig. 1A), induces re-organization of the actin cytoskeleton (Fig. 1B), activates RhoA (Fig. 3) and that this relays to the induction of the transcription factor NF-κB (Figs. 7 and 8) via a signaling cascade that is regulated by PKD1 (Figs. 1C and 2–6).

NF-κB is a sensor for actin re-organization in response to mechanical stresses at the plasma membrane [Ren et al., 1999; Nemeth et al., 2004 and Supplemental Fig. 1]. Recently, it was suggested that RhoA activation induces NF-κB, but mechanisms so far have not been provided [Montaner et al., 1998]. Our data now provide a potential mechanism for this signaling. We show that in response to a calcium switch RhoA activates the serine/threonine kinase PKD1 (Figs. 1B,C and 2 and 3) and that RhoA activates NF-κB through PKD1-mediated signaling.

RhoA regulates many of its downstream effects via the kinase ROCK [Slater et al., 2001, 2003]. We found that ROCK contributes to PKD1 activation in response to loss of cell–cell contacts and expression of active RhoA (Fig. 6). Our data implicate that ROCK is upstream of PKD1 and regulates its tyrosine phosphorylation by SFKs (Figs. 4 and 6). Members of the Src family of cytoplasmatic tyrosine kinases are often found at E-cadherin-based cell–cell contacts of epithelial cells [Tsukita et al., 1991; Calautti et al., 1998]. In general, the roles of v-Src, c-Src, and SFKs at adherens junctions remain poorly understood. Constitutively active v-Src perturbs the integrity of cell–cell interactions by phosphorylating β-catenin [Matsuyoshi et al., 1992]. On the other hand, c-Src and SFKs are necessary for the integrity of E-cadherin-mediated cell–cell contacts and positively affect cell adhesion [Calautti et al., 1998; McLachlan et al., 2007]. McLachlan et al. [2007] recently provided evidence, showing that Src positively supports cadherin function at lower signal strengths and perturbing functions at higher levels. Thus, dependent on signal intensity, Src activity can have different functional outcomes on cell–cell contacts. Our data suggest that SFKs contribute to PKD1 activation when adherens junctions are disrupted. One possible mechanism of how SFK is activated is that the loss of cell–cell contacts releases the SFK from the cadherin junction, leading to its activation and eventually to the phosphorylation of PKD1.

We have shown previously that SFK-mediated tyrosine phosphorylations subsequently lead to the nPKC-mediated activation-loop phosphorylation and activation of PKD1 [Storz and Toker, 2003; Storz et al., 2003, 2004a,b]. For example, in oxidative stress signaling, PKD1-mediated activation of NF-κB is regulated by the SFK member Src and the nPKC family member PKCδ through complex temporal phosphorylation events [Doppler and Storz, 2007]. The nPKC-mediated phosphorylation of PKD at its activation-loop serines has previously been shown to fully correlate with PKD1 activity [Waldron et al., 1999; Waldron and Rozengurt, 2003]. We show here that RhoA induces activation-loop phosphorylation of PKD1 via PKCε (Figs. 3B–D and 4–6). The association of PKC and RhoA was shown in endothelial cells [Chang et al., 1998; Hippenstiel et al., 1998; Slater et al., 2001, 2003], and RhoA has been implicated in PKC activation [Exton, 1999; Oude Weernink et al., 2000; Yuan et al., 2001]. This suggests that in response to RhoA activation, a similar mechanism as previously described for oxidative stress-mediated activation of PKD1 leads to the induction of NF-κB. Both upstream kinases for PKD1 in this pathway, SFK and nPKC, are important regulators of the cytoskeleton and have been implicated in actin re-organization and cell adhesion [Vitale et al., 1992; Kim et al., 2008].

Our data provide a link between loss of cell–cell contacts, actin re-organization, RhoA activation, and subsequent regulation of genes through the inducible transcription factor NF-κB (Fig. 8). But what is the consequence of RhoA-mediated regulation of NF-κB? The transcription factor NF-κB regulates both, adhesion genes and survival genes [Yan et al., 2005; Storz et al., 2005a; Liu et al., 2006]. RhoA is important for actin filament organization, cell–cell adhesion, and cell adhesion to substrate [Slater et al., 2001, 2003; Prahalad et al., 2004; Vielkind et al., 2005]. Thus, one function of RhoA-mediated activation of NF-κB may be the upregulation of

adhesion proteins such as cadherins or integrins. This would implicate a protective mechanism that contributes to the induction of genes which re-establish adherens junctions or maintain epithelial structures. However, the activation of NF-κB following detachment has also been shown to delay apoptosis in intestinal epithelial cells [Yan et al., 2005]. Thus, another possibility is that RhoA-mediated NF-κB activation in response to loss of cell–cell contacts induces genes that increase cell survival of detached cells and thus, for example, may contribute to a malignant tumor phenotype by protecting migrating tumor cells that potentially can form metastases. This will be evaluated in future work.

Taken together, in the above research we show that the disruption of adherens junctions by calcium depletion leads to actin re-organization, activation of RhoA, and RhoA-mediated activation of NF-κB (Fig. 9). We identify PKD1 as a RhoA downstream target that relays RhoA activation to the induction of NF-κB. Further, our data implicate that this signaling pathway is regulated via ROCK, SFK, and nPKC enzymes. We propose that the here-described mechanism is of importance for the re-establishment or maintenance of adherens junctions and epithelial cancer cell survival.

MATERIALS AND METHODS

CELL LINES, ANTIBODIES, AND REAGENTS

HeLa and MCF-7 cell lines were from the American Type Culture Collection (ATCC) and were maintained in high glucose DMEM supplemented with 10% fetal bovine serum. Anti-RhoA antibody, anti-PKCε, anti-PKCδ, anti-PKCη, anti-PKCθ antibodies, and anti-pan-PKD (anti-PKD1-C20 recognizes PKD1 and PKD2) were from Santa Cruz (Santa Cruz, CA), anti-Src from Upstate Biotechnology (Waltham, MA), mouse anti-E-cadherin (human) from Zymed Laboratories (Carlsbad, CA), and anti-HA were from Sigma (St. Louis, MO). Anti-pS744/748-PKD antibody (recognizes pS738/742 in human PKD1) was from Biosource/Invitrogen (Carlsbad, CA). Anti-pY463-PKD was from A. Toker. The rabbit polyclonal anti-pY95-PKD antibody was generated in the laboratory and has been described before [Doppler and Storz, 2007]. A rabbit polyclonal antibody specific for PKD1 was raised against an H2N-MAECQNDS-GEMQDP-amide peptide (amino acids 372–385 in human PKD1). The secondary HRP-linked anti-mouse or anti-rabbit antibodies were from Roche (Indianapolis, IN), the secondary goat anti-rabbit Alexa Fluor 488 F(ab′)2 antibodies from Molecular Probes/Invitrogen (Carlsbad, CA). PKD substrate peptide was described before [Storz et al., 2003]. Rhodamine–Phalloidin and Cytochalasin D (Supplemental Fig. 1) were from Sigma. The ROCK inhibitor Y27632 and the SFK inhibitor PP2 were from Calbiochem/EMD Biosciences (San Diego, CA). Cell culture-grade EDTA solution (0.5 M) was from Cellgro/Mediatech (Manassas, CA). H_2O_2 was from Fisher Scientific (Suanee, GA). *Trans*IT HeLa Monster reagent (Mirus, Madison, WI) or Superfect reagent (Qiagen, Valencia, CA) were used for transient transfections.

DNA CONSTRUCTS

Expression plasmids for wild-type PKD1 and the constitutive-active PKD1 alleles PKD1.S738/742E and PKD1.Y463E are based on an amino-terminal HA-PKD1 cDNA cloned in pcDNA3 using *Bam*HI and *Xho*I sites and have been described elsewhere [Storz et al., 2003; Storz and Toker, 2003]. Expression constructs for active Rho (Rho.V14, Rho.CA), active Rac (Rac.CA), and active cdc42 (Cdc42.CA) were from Dr. P. Z. Anastasiadis, active RhoC from Dr. Keith Burridge, and dominant-negative RhoA (RhoA.DN) and RhoB (RhoB.DN) from Dr. J. A. Copland. Specific PKD1-RNAi, PKD2-RNAi, PKCε-RNAi, and PKCδ-RNAi expression constructs based on pSuper have been described and extensively characterized in previous publications [Storz and Toker, 2003; Storz et al., 2004b, 2005b; Hausser et al., 2005].

IMMUNOBLOTTING AND IMMUNOPRECIPITATION

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 Cocktail (Sigma–Aldrich, St. Louis, MO). Lysates were used for immunoblotting analysis (SDS–PAGE, transfer to nitrocellulose). For immunoprecipitations, lysates were incubated with the respective antibody (2 μg) directed against the protein of interest, followed by a 30-min incubation with protein G-Sepharose (Amersham/Pharmacia, Piscataway, NJ). Immune complexes were washed three times with ice-cold TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl), and subjected to a kinase assay or resolved by SDS–PAGE.

KINASE ASSAYS

PKD1 was immunoprecipitated (anti-PKD1 antibody) and precipitates were washed twice with kinase buffer (50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM dithiothreitol) and then re-suspended in 20 μl kinase buffer. The kinase reaction was carried out for 20 min after the addition of 10 μl of kinase substrate mixture (100 μM PKD-specific substrate peptide, 50 μM ATP, 10 μCi [γ-32P] ATP in kinase buffer). To terminate the assay, the samples were centrifuged and the supernatants spotted onto P81 phosphocellulose paper (Whatman, Clifton, NJ). The papers were washed three times with 0.75% phosphoric acid, once with acetone, dried, and the activity was determined by liquid scintillation counting. To measure PKD1 autophosphorylation, the kinase reaction was performed without substrate peptide and the samples were then separated on a SDS–PAGE, transferred to nitrocellulose, and analyzed with a phosphoimager (Typhoon 9410; Amersham Biosciences).

Rho ACTIVITY ASSAYS

Cells were lysed and GST-Rhotekin pull-down assays were performed as previously described [Simpson et al., 2004]. Active RhoA was detected using an anti-RhoA antibody.

RNA INTERFERENCE (RNAi)

In all experiments where RNAi was employed to silence gene expression, either the control vector (pSuper) or specific RNAi constructs were transfected using *Trans*IT HeLa Monster reagent 12 h after plating. Twenty-four hours after the first transfection with RNAi, the remaining constructs (i.e., NF-κB reporter) were transfected in a second transfection using Superfect. Cells were analyzed 48 h after the initial transfection.

IMMUNOFLUORESCENCE MICROSCOPY

Cells were plated on glass coverslips at a density of 60,000 cells/well in a 24-well plate. Following stimulation, samples were washed twice with PBS and fixed in 3.5% paraformaldehyde (15 min, 37°C). Following fixation, cells were washed three times in PBS and then permeabilized with 0.1% Triton X-100 in PBS for 2 min at RT. Samples were blocked with 3% bovine serum albumin and 0.05% Tween 20 in PBS (blocking solution) for 30 min at RT. The coverslips were then incubated with primary antibody diluted to 1:2,000 in blocking solution for 2 h at RT. Cells were washed five times with PBS and incubated with the secondary antibody diluted to 1:500 in blocking solution (goat anti-rabbit Alexa Fluor 488 F(ab′)2) for 2 h at RT. F-actin staining was performed together with secondary antibody staining by incubating with Rhodamine–Phalloidin. After extensive washes in PBS, coverslips were mounted in Fluormount-G (Southern Biotech, Birmingham, AL). Samples were examined using an Olympus IX71 fluorescence microscope or an LSM 510META confocal laser-scanning microscope (Zeiss, Jena, Germany) with a Plan-Apochromat 63×/1.4 Dic oil immersion objective. Alexa Fluor 488 was excited with the 488 nm laser and Rhodamine–Phalloidin at the 543 nm line. Images were acquired in multi-track configuration, switching tracks after each line with filters BP505-530 for Alexa Fluor 488, and META channels 560-635 for Rhodamine. Images shown depict single confocal sections. Images were processed using NIH ImageJ.

REPORTER GENE ASSAYS

Cells were transiently co-transfected with an NF-κB-reporter construct (NF-κB-luc, 3 μg), pCMV-renilla-luc (0.1 μg), and the protein(s) of interest (1 μg) using Superfect (Qiagen). Twenty-four hours after transfection, lysates were prepared by washing cells twice with ice-cold PBS, scraping in 250 μl passive lysis buffer (Promega), and centrifuging (13,000 rpm, 10 min, 4°C). Assays for luciferase activity were performed on total cell lysates in a 96-well plate according to the Promega Dual-Luciferase assay protocol and measured using a Veritas luminometer (Symantec, Cupertino, CA). Luciferase activity of the NF-κB-reporter construct was normalized to *Renilla* luciferase activity. Protein expression was controlled by immunoblot analysis.

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