Protein Kinase D Regulates RhoA Activity via Rhotekin Phosphorylation*

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Background: The substrates of protein kinase D (PKD) that regulate actin cytoskeletal reorganization are largely unknown.
Results: Rhotekin is a novel PKD substrate that regulates RhoA activity by enhancing its membrane association.
Conclusion: PKD-mediated rhotekin phosphorylation demonstrates a novel mechanism of RhoA activation and actin stress fiber formation.
Significance: This study contributes to our understanding of the mechanism of RhoA activation and actin cytoskeletal reorganization regulated by PKD.

The members of the protein kinase D (PKD) family of serine/threonine kinases are major targets for tumor-promoting phorbol esters, G protein-coupled receptors, and activated protein kinase C isoforms (PKCs). The expanding list of cellular processes in which PKDs exert their function via phosphorylation of various substrates include proliferation, apoptosis, migration, angiogenesis, and vesicle trafficking. Therefore, identification of novel PKD substrates is necessary to understand the profound role of this kinase family in signal transduction. Here, we show that rhotekin, an effector of RhoA GTPase, is a novel substrate of PKD. We identified Ser-435 in rhotekin as the potential site targeted by PKD *in vivo*. Expression of a phosphomimetic S435E rhotekin mutant resulted in an increase of endogenous active RhoA GTPase levels. Phosphorylation of rhotekin by PKD2 modulates the anchoring of the RhoA in the plasma membrane. Consequently, the S435E rhotekin mutant displayed enhanced stress fiber formation when expressed in serum-starved fibroblasts. Our data thus identify a novel role of PKD as a regulator of RhoA activity and actin stress fiber formation through phosphorylation of rhotekin.

The protein kinase D (PKD)[6] family of serine/threonine kinases belongs to the calcium/calmodulin-dependent protein kinase superfamily (1, 2) and comprises three members: PKD1/PKCμ (3, 4), PKD2 (5), and PKD3/PKCν (6). PKDs are activated either directly via phorbol esters or indirectly by various mechanisms, including G protein-coupled receptors (7). At the structural level, the N-terminal region comprises the regulatory cysteine-rich zinc finger domain and a pleckstrin homology domain, whereas the C-terminal half constitutes the catalytic kinase domain (2). Depending on their activation status and the cell type examined, these individual domains of PKDs regulate their localization to different compartments of the cell, including the cytosol, nucleus, mitochondria, plasma membrane, and Golgi complex (8). PKDs were found to be involved in various cellular processes. The functions of PKDs depend on the stimulus and the subcellular localization of the respective isoform. Various compartment-specific functions have been shown for PKDs (8). Although the majority of the functions were described for PKD1, the other two members (*i.e.* PKD2 and PKD3) were also found to be involved in multiple cellular processes, including proliferation, adhesion, migration, apoptosis, cell survival, transcriptional activation, cardiac hypertrophy, immune responses, angiogenesis, Golgi organization, vesicle trafficking, and hormone secretion (8–15). Although the three isoforms display certain amount of redundancy with respect to their function, there are at the same time unique functions that can be attributed to each isoform (16, 17). The functional outcome of a PKD-mediated cellular pathway arises from either direct substrate phosphorylation or association of substrates to additional kinases and adaptors. Thus, the identification of novel substrates is a prerequisite to understand the critical role of this kinase family in various biological processes.

Rhotekin literally means "Rho target" (from the Japanese "teki," meaning target), and the protein was identified in yeast

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[6] The abbreviations used are: PKD, protein kinase D; PMA, phorbol 12-myristate 13-acetate; DA, D695A; SSEE, S706E/S710E; PI4Kβ, phosphatidylinositol 4-kinase IIIβ; Ab, antibody; RBD, Rho binding domain.

two-hybrid screens as a Rho interactor (18). It is classified together with rhophilin and protein kinase N as a class I Rho binding domain-containing protein. Rhotekin has been suggested to sequester Rho in its active form and inhibit RhoGAP-stimulated or endogenous Rho GTPase hydrolysis (19). The subcellular functions of rhotekin are not well understood. High rhotekin expression has been correlated with an advanced stage of gastric, colorectal, and bladder cancer and has been shown to mediate NF-κB activation, thereby conferring resistance to apoptosis (20, 21). Rhotekin was shown to interact with septin9b and to colocalize with septin9b and stress fibers upon lysophosphatidic acid treatment of rat embryonic fibroblast cells (22). In addition, rhotekin interacts with PDZ domain-containing proteins like TIP-1 and PIST and also with a cell polarity-related protein, Lin7b. The latter interaction was found to be regulated by Rho (23–25). Rhotekin was also shown to interact with a multidomain adaptor protein, vinexin, with a possible role at focal adhesion formation (26).

In the present study, we have identified the class I Rho binding domain-containing protein, rhotekin, as a novel substrate of PKD. We show that all of the PKD isoforms can phosphorylate rhotekin *in vitro*. Using mass spectrometry, we have identified Ser-435 in the C terminus of rhotekin as the potential site targeted by PKD. We generated a phosphosite-specific antibody that specifically detects phosphorylation of rhotekin at Ser-435, and using this antibody, we confirmed that this site is indeed a PKD target *in vivo*. Endogenous activity of RhoA was increased upon overexpression of a phosphomimicking S435E rhotekin mutant in HEK-293T cells. In addition, PKD2-mediated phosphorylation of rhotekin increased and dephosphorylation decreased the anchoring of RhoA in the plasma membrane. Consequently, there was an increase in actin stress fiber formation in the presence of S435E rhotekin mutant compared with cells expressing rhotekin wild type or a nonphosphorylatable Ser[435]Ala mutant. In conclusion, our data show a novel mechanism of RhoA activation and actin stress fiber formation by PKD-mediated rhotekin phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK-293T and NIH-3T3 cells were grown in DMEM with 10% (v/v) FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 10% CO_2 at 37 °C. Exponentially growing NIH-3T3 cells were transfected with Lipofectamine LTX (Invitrogen). HEK-293T cells were grown to 50% confluence and transfected with polyethyleneimine (Polysciences Inc.).

Antibodies and Reagents—Anti-GFP antibody was purchased from Roche Applied Science, anti-Myc tag antibody was from Cell Signaling, and anti-FLAG and anti-β-actin antibodies were from Sigma. Antibody that detects the phosphorylated PKD at Ser-744/748 was from Cell Signaling. Anti-rhotekin antibody was from Novus Biologicals, anti-RhoA antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-PKD2 antibody was from Bethyl Laboratories Inc. Alexa Fluor 488-labeled anti-mouse and Alexa Fluor 568-labeled phalloidin were purchased from Molecular Probes. [γ-^{32}P]ATP (5000 Ci/mmol; 37 GBq = 1 mCi) was purchased from GE Healthcare. Phorbol 12-myristate 13-acetate (PMA)

was purchased from Calbiochem. All other reagents were of the highest grade available.

DNA Constructs—GFP-PKD1, EGFP-PKD2 WT, EGFP-PKD2 D695A (DA), EGFP-PKD2 S706E/S710E (SSEE), and GFP-PKD3 have been described previously (27). pRK5-FLAG-rhotekin was a kind gift from Prof. K. Nagata (Kasugai, Japan). It was constructed by PCR and subcloning into FLAG-pRK5 (22). FLAG-rhotekin S435A and S435E were generated by performing a PCR using the QuikChange site-directed mutagenesis system (Stratagene). Myc-rhotekin WT was generated by amplification of rhotekin by PCR with pRK5-FLAG-rhotekin as template and a 5′ sense primer (5′-GCAGGATCCATGCAG-GACAGATTG-3′) containing a BamHI site and a 3′ antisense primer (5′-CCCAGCTTTCTTGTCGACAGTGG-3′) containing a SalI site. The fragment was cloned into BamHI- and SalI-digested pCMV-Tag 3B vector (Stratagene). GST-RBD was a kind gift from Prof. Martin Schwartz (University of Virginia, Charlottesville, VA). pEGFP-RhoA was kindly provided by Prof. K. Giehl (Giessen, Germany).

Substrate Identification Algorithm—We utilized Scansite (available from the MIT Web site), which is a publicly available substrate prediction Web tool. Scansite utilizes a position-specific scoring matrix to predict substrates for protein kinases. Because the database search with Scansite resulted in a list of 500 proteins that could serve as potential PKD substrates, we applied additional filters to increase the selectivity of our screen. The first criterion we applied was the prerequisite that PKD substrates must have an Arg/Lys (a basic amino acid) at position P−3 and a hydrophobic amino acid at position P−5 (preferably Leu, Ile, or Val). A preference for an arginine/lysine at position P−3 is a requirement for all kinases from the calcium/calmodulin-dependent protein kinase group. The strong preference for a hydrophobic amino acid at P−5 was deduced from the oriented peptide library approach, and its presence was observed in all of the physiological PKD substrates that are known thus far (28). A second point of selection was to find out if the potential phosphorylation sites were evolutionarily conserved. For this purpose, protein sequences were compared by BLAST in different organisms, and conservation of the site between *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus* was taken as an additional selection criterion. The final selection criterion included was the consideration of Ser/Thr exposure toward the surface of the substrate of interest. Although in Scansite, a surface accessibility plot is generated for each protein, we excluded this option because this calculation is done based on the primary sequence of proteins. We tried to derive information on surface accessibility from the available crystal structures or used modeling approaches for substrates where structural details were not known. The modeling approach was done using 3DPSSM version 2.6.0 (available from the Structural Informatics Group Web site), and structures were visualized using Rasmol version 2.7.2.1 (available on the World Wide Web). The position of the phosphorylation site in secondary structures was also evaluated using Predict Protein (available on the World Wide Web). This resulted in the identification of novel PKD substrates, one of them being rhotekin. It is worth mentioning that RIN1 and CREB, known substrates of PKD1,

were retrieved as well from the database after our multicriterion search.

Immunoprecipitation and Western Blotting—Immunoprecipitations and Western blotting were performed as described previously (27). Briefly, transfected HEK-293T cells were lysed in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 8.8, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2.5 mM MgCl$_2$, protease and phosphatase inhibitor mixture (Roche Applied Science)). After centrifugation at 12,000 × *g* for 10 min, protein concentrations were measured in the lysates. 2000 μg of extracts were precleared with protein A-Sepharose beads (GE Healthcare) at 4 °C for 30 min. The precleared extracts were incubated with the primary antibody (2 μg) at 4 °C, and after 1 h, 30 μl of protein A-Sepharose beads were added and incubated for 1 h. Immobilized proteins were washed extensively and used for either *in vitro* kinase assay or resuspended in Laemmli buffer and subjected to SDS-PAGE. The gels were blotted onto a PVDF membrane and blocked with 5% milk or BSA (for Ser(P)-435 rhotekin antibody) in 0.1% TBS-Tween buffer (TBS-T). Incubation with the primary antibodies was performed in TBS-T for 1 h at room temperature. After washing with TBS-T, samples were incubated with secondary horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG antibodies in TBS-T for 1 h at room temperature. Detection was performed with enhanced chemiluminescence (ECL). Band intensities were quantified using Bioprofil BIO-1D software (version 12.04).

In Vitro Kinase Assay—An *in vitro* kinase assay was performed as described previously (27). Briefly, to examine the *in vitro* rhotekin phosphorylation by PKDs and their mutants, HEK-293T cells expressing GFP-tagged PKDs or PKD2 mutants were left either stimulated (+) or unstimulated (−) with PMA (400 nM, 10 min) and lysed in lysis buffer I (50 mM Tris-HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol (DTT), 1% Triton X-100, and protease and phosphatase inhibitor mixture). Anti-GFP immunoprecipitates were prepared as described above, and immune complexes were washed with lysis buffer I, followed by lysis buffer II (buffer I without Triton X-100) and finally with kinase buffer (30 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, and 1 mM DTT). The immune complexes were then resuspended in 20 μl of kinase buffer in the presence of purified rhotekin and 100 μM [γ-^{32}P]ATP. Reactions were incubated for 10 min at 30 °C, terminated by adding an equal amount of 2× Laemmli buffer, and analyzed by SDS-PAGE, followed by exposure onto a phosphor screen. Radioluminographs were developed using a Fujifilm BAS scanner (Fuji Photo Film).

Identification of PKD-mediated Phosphorylation Site in Rhotekin by Mass Spectrometry—Radioactively phosphorylated rhotekin obtained through an *in vitro* kinase assay was subjected to SDS-PAGE and transferred to nitrocellulose membrane (PROTRAN) in the Hoefer SE600 electrophoresis and blotting system. Protein bands were visualized with Coomassie Brilliant Blue staining and excised for in-gel trypsinization. Therefore, the gel slice was incubated for 18 h at 37 °C with sequence grade trypsin (Roche Applied Science) in 200 nM ammonium bicarbonate, pH 8.0. Resulting peptides were

extracted with 60% acetonitrile in HPLC water (Labscan). They were dried in a Savant SpeedVac concentrator. Peptides were solubilized again in 0.1% trifluoroacetate in HPLC water and separated on a C2/C18 reverse phase column (GE Healthcare). Peak fractions were collected manually. The position of radioactive labeled peptides was determined by analysis of each fraction with a Wallac 1409 Liquid Scintillation counter. Further purification of the peptide fractions was performed using an Fe^{2+}-coupled IMAC. The purified peptide sample was analyzed with ESI-MS on a SCIEX API-3000 triple quadrupole mass spectrometer.

Generation of Phosphosite-specific Antibody—The phosphopeptide for immunization (AKQGpSLYHE) was coupled with keyhole limpet hemocyanin (Pierce) and BSA through glutaraldehyde. The peptide-keyhole limpet hemocyanin conjugates were used to immunize rabbits. Antibodies were precipitated with 50% ammonium sulfate from rabbit serum and redissolved in PBS. Phosphospecific antibody was purified sequentially on a dephosphopeptide column and a phosphopeptide column. This was prepared by binding BSA-coupled peptides on CNBr-activated Sepharose 4-B according to the manufacturer's instructions (GE Healthcare). Elution of antibody from the phosphopeptide column was done with 100 mM glycine, pH 3.0. Eluates were immediately neutralized with 1 M Tris-Cl, pH 8. Purified antibody was tested on ELISA. Briefly, BSA-coupled phospho- and dephosphopeptides were coated on a microtiter plate (100 μl of 10 μg/ml solution in PBS per well) overnight at 4 °C. Wells were washed three times with buffer A (PBS, 0.5 M NaCl, 0.1% Tween 20, pH 7.2) and incubated with 100 μl of primary antibody (dilutions ranging from 1:250 to 1:128,000 in buffer A plus 3% PEG 6000) for 2 h at room temperature. After washing three times with Buffer A, incubation with secondary anti-rabbit HRP-conjugated antibody (diluted in buffer A plus 3% PEG 6000) was done for 1–2 h at room temperature. This was followed by a washing step as described above, and colorimetric detection was done with 3,3′,5,5′-tetramethylbenzidine as a substrate at 450 nm in a spectrophotometer.

Rho GTPase Activity Assay—HEK-293T cells were transfected with either Myc-empty vector or Myc-tagged rhotekin WT or mutants. 24 h after transfection, the growth medium was replaced with serum-free medium. 18 h after serum starvation, the cells were washed with ice-cold PBS buffer and lysed in radioimmunoprecipitation assay buffer. Cell lysates were clarified by centrifugation at 14,000 rpm at 4 °C for 10 min, and 2000 μg of lysates were incubated with 20 μg of GST-RBD beads immobilized on glutathione-Sepharose beads (GE Healthcare) at 4 °C for 2 h. Beads were washed extensively, resuspended in Laemmli buffer, and analyzed for bound active endogenous RhoA by SDS-PAGE and Western blotting. The quantification of GTP-bound RhoA (active RhoA) for different samples was calculated by normalizing the band intensity of GST-RBD-bound RhoA to total RhoA in cell lysates.

Subcellular Fractionation of RhoA—HEK-293T cells were seeded at 95% density in 6-well plates and transfected with the indicated constructs using polyethyleneimine transfection reagent. Cells were harvested and lysed in 500 μl of cellular fractionation buffer (250 mM sucrose, 20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA and EGTA, 1 mM DTT) sup-
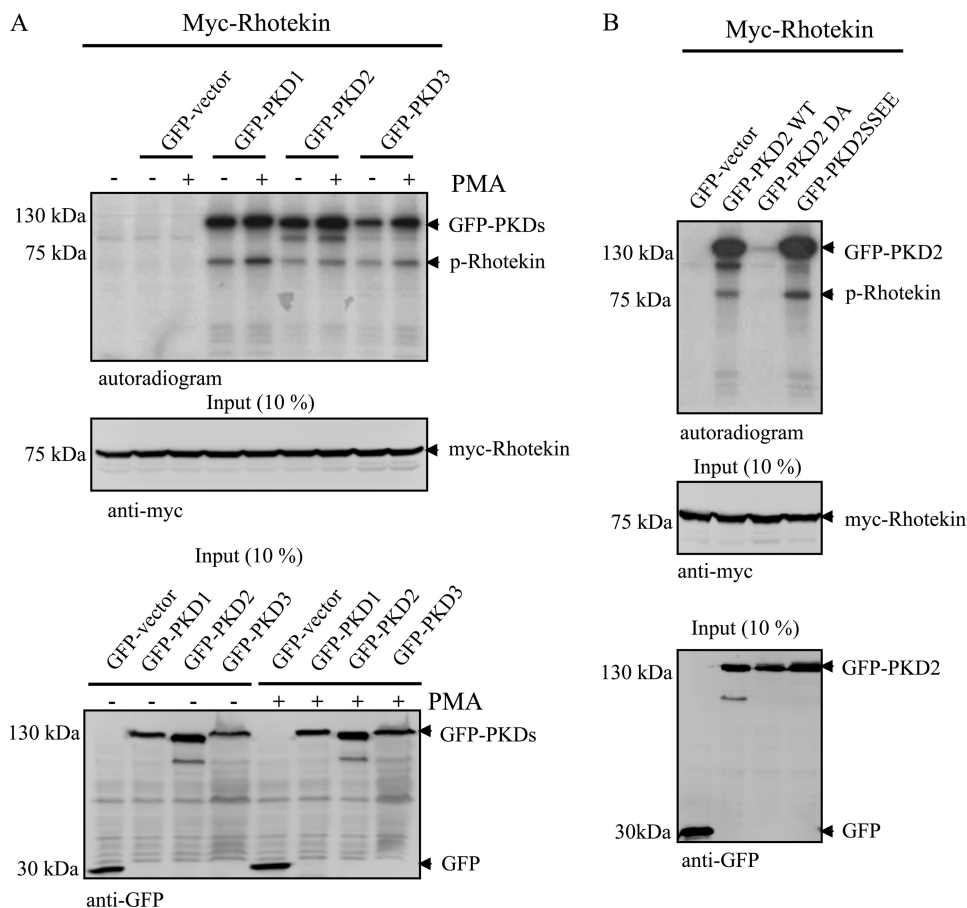
FIGURE 1. **PKDs phosphorylate rhotekin *in vitro*.** *A*, HEK-293T cells were transfected with EGFP-empty vector, GFP-PKD1, EGFP-PKD2, or GFP-PKD3. 36 h after transfection, cells were incubated with 400 nM PMA for 10 min as indicated. Cells were subsequently lysed, and lysates were subjected to immunoprecipitation with GFP antibody. Aliquots of the immunoprecipitates were analyzed by Western blotting using the GFP antibody to determine the expression level of the three PKD isoforms. Immunoprecipitates were further subjected to an *in vitro* kinase assay with equal amounts of purified Myc-rhotekin in all of the conditions described under "Experimental Procedures." *B*, HEK-293T cells were transfected with EGFP-empty vector, wild-type PKD2 (*EGFP-PKD2 WT*), kinase-dead PKD2 (*EGFP-PKD2 DA*), or constitutively active PKD (*EGFP-PKD2 SSEE*). 36 h after transfection, cells were subsequently lysed, and lysates were subjected to immuno-precipitation with GFP antibody. Aliquots of the immunoprecipitates were analyzed by Western blotting using the GFP antibody to determine the expression level of WT PKD2 and the mutants. Immunoprecipitates were further subjected to *in vitro* kinase assay with equal amount of purified Myc-rhotekin in all of the conditions described under "Experimental Procedures." Representative blots from three independent experiments are shown. *p-Rhotekin*, phosphorhotekin.

plemented with Complete protease inhibitor mixture and PhosStop (Roche Applied Science) by passing them through a 25-gauge syringe needle 15 times. Lysates were clarified by centrifugation at $10,000 \times g$ for 20 min. Equal amounts of total protein were subjected to $100,000 \times g$ ultracentrifugation to separate cytoplasm and membrane fractions. Membrane fractions were washed two times with cellular fractionation buffer followed by ultracentrifugation for 1 h. Membrane pellets were dissolved in radioimmunoprecipitation assay buffer, 10% glycerol, and equal amounts of membrane and cytoplasm fractions were resolved on 10% SDS gels followed by Western blotting. National Institutes of Health ImageJ calculated relative band intensities following densitometry.
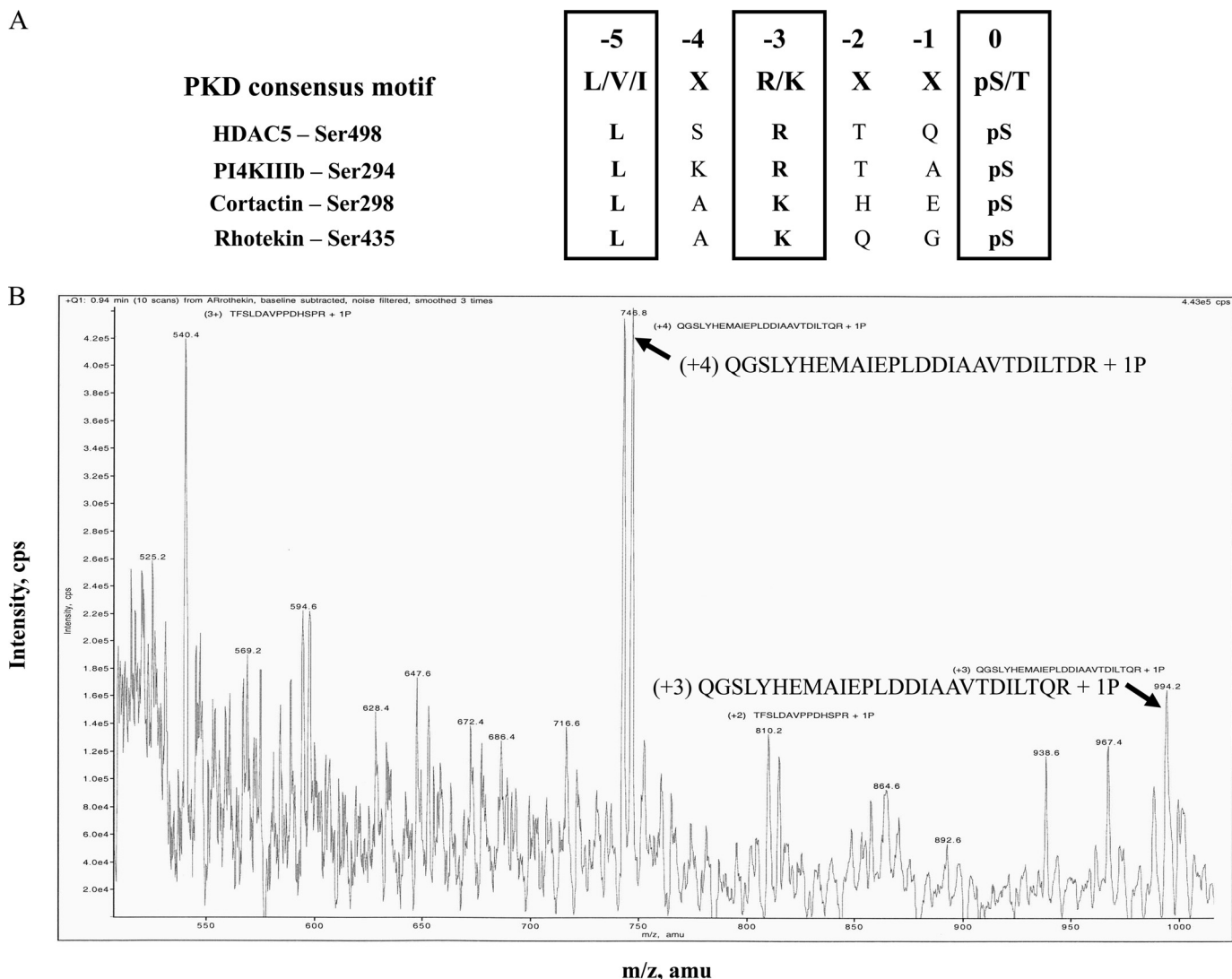
Immunofluorescence Microscopy—NIH-3T3 cells were seeded on glass coverslips coated with fibronectin (5 μg/cm^2; Roche Applied Science) and transfected the following day. 6 h after the transfection, the growth medium was replaced with serum-free medium. 18 h after serum starvation, the cells were fixed with 4% formaldehyde in PBS for 10 min, blocked, and permeabilized with blocking buffer (0.05% saponin and 1% BSA in PBS) for 30 min. The coverslips were incubated with primary

antibody diluted in blocking buffer for 1 h at room temperature, washed, and incubated with secondary antibody conjugated to Alexa Fluor 488 dye, diluted in blocking buffer for 1 h (Alexa Fluor 568-phalloidin, to visualize F-actin; Hoechst, to stain DNA, was added during the secondary antibody step), washed, and mounted using Fluor Save reagent (Calbiochem). Imaging was performed with a Keyence inverted fluorescence microscope with filters detecting excitations at 360 (Hoechst), 490 (Alexa Fluor 488), and 560 (Alexa Fluor 568) and a $\times 60$ oil objective. Images were processed using ImageJ.

Statistics—The statistical significance of the difference between means was determined using the two-tailed Student's *t* test. Differences were considered significant at $p < 0.05$.

RESULTS

Rhotekin Is a Novel Substrate of PKDs—To identify potential PKD substrates, we employed an *in silico* approach using the publicly available Scansite Web tool, a bioinformatics tool used for predicting potential phosphorylatable substrates of various kinases. After obtaining several candidate PKD substrates, we have utilized additional criteria to filter the list of substrates,

A

	-5	-4	-3	-2	-1	0
PKD consensus motif	**L/V/I**	**X**	**R/K**	**X**	**X**	**pS/T**
HDAC5 – Ser498	**L**	S	**R**	T	Q	**pS**
PI4KIIIb – Ser294	**L**	K	**R**	T	A	**pS**
Cortactin – Ser298	**L**	A	**K**	H	E	**pS**
Rhotekin – Ser435	**L**	A	**K**	Q	G	**pS**

B

FIGURE 2. **Identification of Ser-435 in rhotekin as the critical phosphorylation site targeted by PKDs.** *A*, alignment of the consensus PKD substrate motif of the known PKD substrates, namely HDAC5, PI4Kβ, and cortactin. The PKD substrate consensus motif contains Arg (or at least a basic amino acid, such as Lys) at position P−3 and a hydrophobic amino acid at position P−5 (preferably Leu, Ile, or Val). Ser-435 in rhotekin fulfills the criteria of ideal PKD phosphorylation consensus sequence. *B*, rhotekin obtained in the *in vitro* kinase assay was purified and subjected to mass spectrometry as described under "Experimental Procedures." MS spectrum of trypsin-digested, IMAC-enriched rhotekin is depicted here. *m/z* 994.2 (3+) and *m/z* 746.8 (4+) (indicated by *arrows*) can be explained as singly phosphorylated [433]QGSLYHEMAIEPLDDIAAVTDILTQR[458] peptide. *amu*, atomic mass units.

including conservation of amino acid residues at positions −5 and −3 relative to the Ser(P) or Thr(P) site, evolutionary conservation, and accessibility of the phosphorylation site (for details, see "Experimental Procedures"). One of the substrates that were identified following the above mentioned criteria was rhotekin. Because PKDs have been implicated in cell migration and invasion (8), we wanted to explore whether rhotekin is indeed an *in vivo* substrate of PKDs and, if so, to establish the biological function of PKD-phosphorylated rhotekin. To validate our *in silico* data, we first performed *in vitro* kinase assays using purified Myc-tagged rhotekin and GFP-tagged PKDs. All three PKD isoforms phosphorylated rhotekin, and the phosphorylation was enhanced in the presence of PMA treatment, 1.4-fold for PKD1, 1.25-fold for PKD2, and 1.3-fold for PKD3 (Fig. 1*A*). In addition, phosphorylation of rhotekin was examined in the presence of PKD2 wild type (WT), kinase-dead (DA), and constitutively active

(SSEE) mutants (27). As shown in Fig. 1*B*, phosphorylation of rhotekin in the *in vitro* kinase assay was observed by wild-type PKD2 and was enhanced upon expression of the constitutively active mutant of the kinase (PKD2 SSEE). In contrast, there was no detectable phosphorylation of rhotekin by a catalytically inactive PKD2 mutant (PKD2 DA). This result confirmed that rhotekin is indeed a genuine *in vitro* substrate of PKDs.

Serine 435 in Rhotekin Is Phosphorylation Site Targeted by PKDs—Having established that rhotekin is a substrate of PKDs, we aimed to identify the phosphorylation site in rhotekin that is targeted by PKDs. Ser-435 was predicted to match the typical PKD substrate consensus motif ([430]LAKQGSpLYHEM[440]) (Fig. 2*A*). Next, we performed mass spectrometry using rhotekin obtained from the *in vitro* kinase assay (for details, see "Experimental Procedures") and correlated the phosphoproteomic data with the Scansite prediction. Indeed, Ser-435 was

A

B
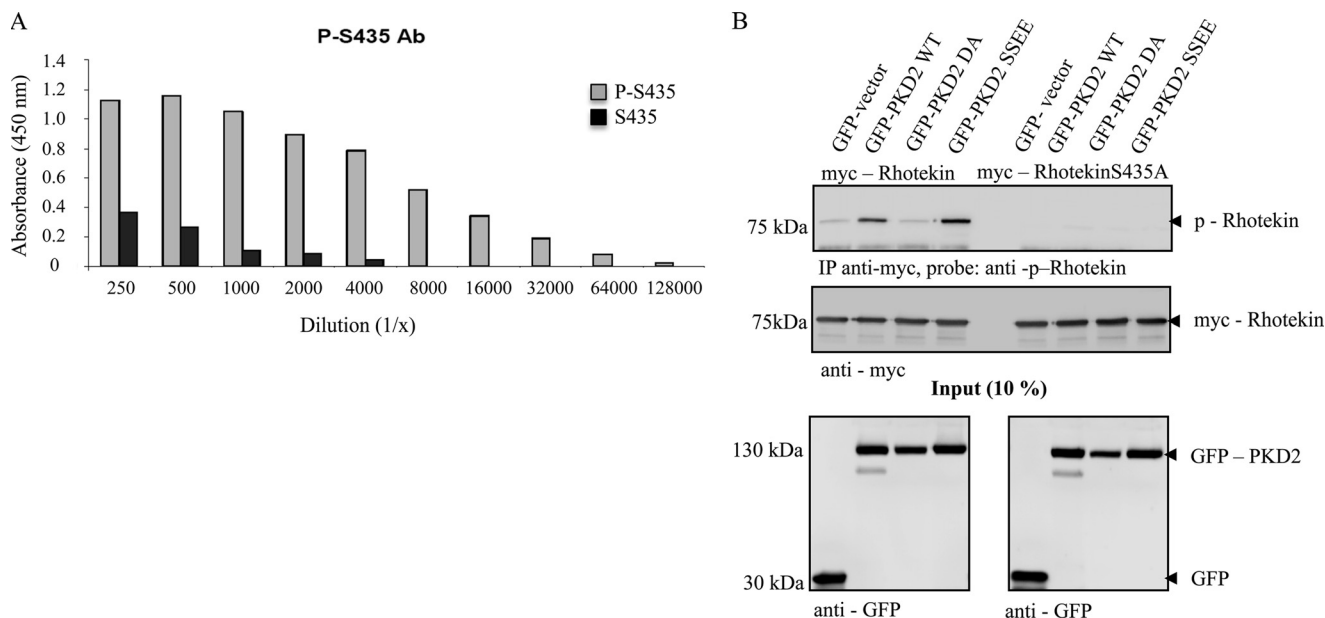
FIGURE 3. **Ser(P)-435 Ab specifically recognizes Ser-435 phosphorylation mediated by PKD.** *A*, ELISA experiment demonstrating the specificity of the Ser(P)-435 antibody. *Gray bars*, absorbance values for AKQGSpLYHE recognition; *black bars*, absorbance values for AKQGSLYHE recognition. *B*, HEK-293T cells were co-transfected with either EGFP-empty vector, wild-type PKD2 (*EGFP-PKD2 WT*), kinase-dead PKD2 (*EGFP-PKD2 DA*), or constitutively active PKD (*EGFP-PKD2 SSEE*) and wild-type rhotekin (*myc-Rhotekin*) or dephosphorylatable mutant (*myc-Rhotekin S435A*). 36 h after transfection, cells were subsequently lysed, and lysates were subjected to immunoprecipitation (*IP*) with Myc antibody and probed with Ser(P)-435 antibody. To verify that both wild-type and dephosphorylatable rhotekin mutant were present in equal amounts in all of the conditions, the blot was stripped and reprobed with Myc antibody. The expression of PKD2 wild-type and mutants was analyzed by Western blotting using the GFP antibody. Representative blots from three independent experiments are shown.

identified as the target residue of active PKDs in rhotekin (Fig. 2*B*).

To further validate these data from mass spectrometry and to determine whether rhotekin was also an *in vivo* substrate of PKDs, we generated a phosphosite antibody that specifically detects phosphorylation of rhotekin at Ser-435 (Ser(P)-435 Ab; for details, see "Experimental Procedures"). The Ser(P)-435 antibody detected a rhotekin peptide phosphorylated at Ser-435 but not the corresponding nonphosphorylated peptide (Fig. 3*A*). Next, we tested this antibody in an *in vivo* scenario. Myc-tagged wild-type rhotekin or a nonphosphorylatable mutant (Myc-rhotekin S435A) was cotransfected with EGFP-empty vector, EGFP-PKD2 wild type, or its respective catalytically inactive (DA) or constitutively active (SSEE) mutant. The expression levels of PKD2 and rhotekin were comparable in all conditions (Fig. 3*B*). Immunoprecipitation of rhotekin from cell lysates followed by blotting with the Ser(P)-435 antibody revealed that there was no rhotekin Ser-435 phosphorylation in cells transfected with rhotekin S435A mutant together with PKD2 WT or mutants, respectively (Fig. 3*B*). In contrast, expression of wild type and in particular constitutively active PKD2 (SSEE) induced striking phosphorylation of rhotekin at Ser-435, whereas catalytically inactive PKD2 (DA) failed to induce rhotekin phosphorylation *in vivo* (Fig. 3*B*). Furthermore, phosphorylation of rhotekin at Ser-435 was prevented when cells were incubated with a selective PKD inhibitor, Gö6976 (data not shown). Thus, Ser-435 is indeed a critical residue targeted by PKD2 *in vivo* (Fig. 3*B*).

To further evaluate phosphorylation of rhotekin at Ser-435, we analyzed the effect of PKD activators and ectopic PKD2 expression on endogenously expressed rhotekin. HEK-293T cells that express both rhotekin and PKDs were incubated with PMA. PMA treatment induced phosphorylation/activation of PKDs as detected using an antibody that detects activation loop phosphorylation of PKDs (8) (Fig. 4*A*, *bottom panels*). Furthermore, there was phosphorylation of endogenous rhotekin at Ser-435 as detected by the Ser(P)-435 antibody (Fig. 4*A*, *top panels*).

To exclude the possibility that the observed rhotekin phosphorylation was not solely dependent on PKDs but due to PMA-induced activation of other kinases, we overexpressed EGFP-tagged PKD2 wild type or mutants and examined the phosphorylation of endogenous rhotekin. Endogenous rhotekin was phosphorylated at Ser-435 only in the presence of wild-type and constitutively active PKD2 but not upon expression of empty vector or catalytically inactive PKD2 (Fig. 4*B*). These data suggest that rhotekin is indeed an *in vivo* substrate of PKDs.

Phosphorylation of Rhotekin at Ser-435 Induces RhoA Activation—Previous studies demonstrated that overexpression of rhotekin confers resistance to apoptosis by activating NF-κB. This effect requires activation of Rho signaling (20). These data prompted us to examine a potential effect of Ser-435 phosphorylation on NF-κB activity. However, luciferase reporter assays showed no significant effect of rhotekin Ser-435 phosphorylation on NF-κB reporter activity (data not shown). Next, we examined the effect of rhotekin Ser-435 phosphorylation on the activation status of RhoA. Overexpression of wild-type rhotekin in HEK-293T cells resulted in a slight increase in the levels of endogenous, GTP-bound RhoA as compared with transfection of the empty vector (Fig. 5*A*). The amount of active RhoA was similar to that of wild-type rhotekin in cells express-

A

B
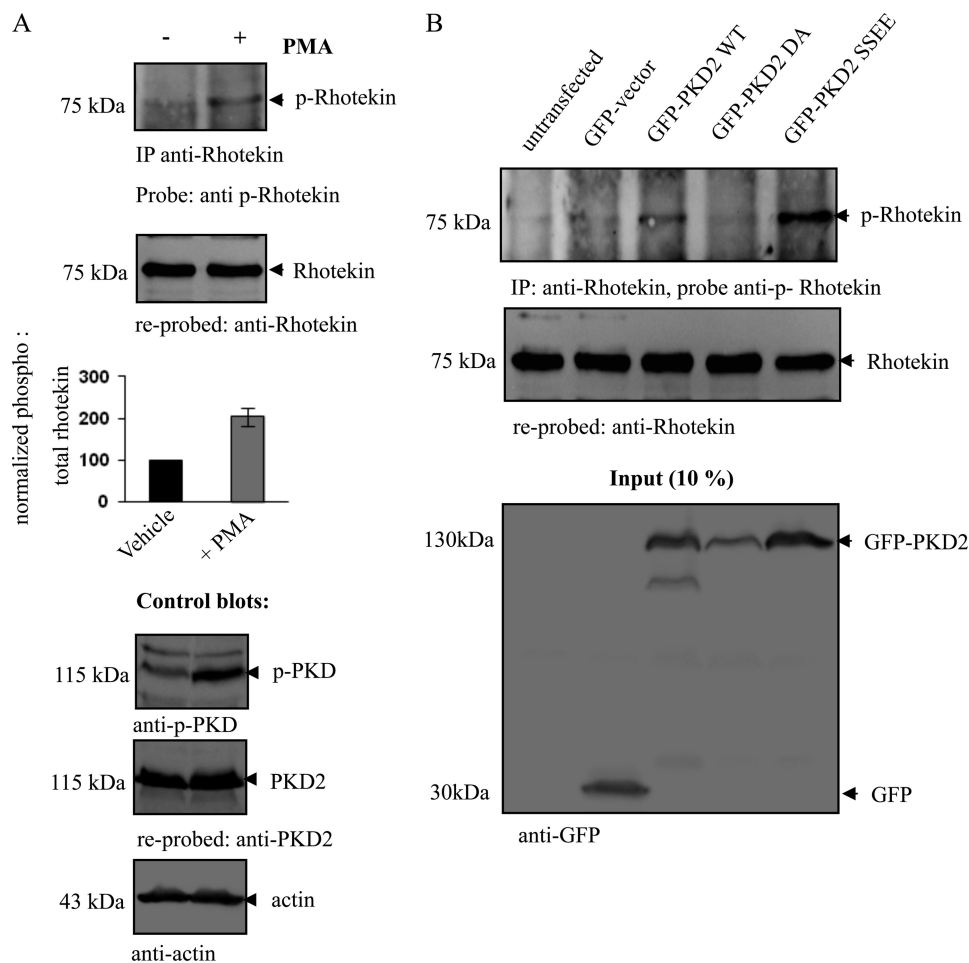
FIGURE 4. **Detection of endogenous rhotekin phosphorylation with Ser(P)-435 Ab.** Subconfluent HEK-293T cells were serum-starved overnight and treated with 400 nM PMA for 10 min as indicated. Cells were subsequently lysed, and lysates were subjected to immunoprecipitation (*IP*) with Myc antibody, followed by probing with Ser(P)-435 Ab to detect endogenous rhotekin phosphorylation. To verify that equal amounts of rhotekin were immunoprecipitated in both conditions, the blot was stripped and reprobed with Myc antibody. Quantification of the band intensities of phosphorhotekin (*p-Rhotekin*) in the cells that were either untreated or treated with PMA is depicted. Phosphorylation of PKDs in the catalytic loop that represents the activation of the kinase was detected by probing with Ser(P)-744/748 antibody. PKD2 and β-actin expression in the lysates used for the immunoprecipitation was monitored as a loading control. *B*, HEK-293T cells were transfected with EGFP-empty vector, wild-type PKD2 (*EGFP-PKD2 WT*), kinase-dead PKD2 (*EGFP-PKD2 DA*), or constitutively active PKD (*EGFP-PKD2 SSEE*). 36 h after transfection, cells were subsequently lysed, and lysates were subjected to immunoprecipitation with Myc antibody, followed by probing with Ser(P)-435 Ab. to detect endogenous rhotekin phosphorylation. Aliquots of the immunoprecipitates were analyzed by Western blotting using the GFP antibody to determine the expression level of WT PKD2 and the mutants. Representative blots from three independent experiments are shown. *Error bars*, S.E.
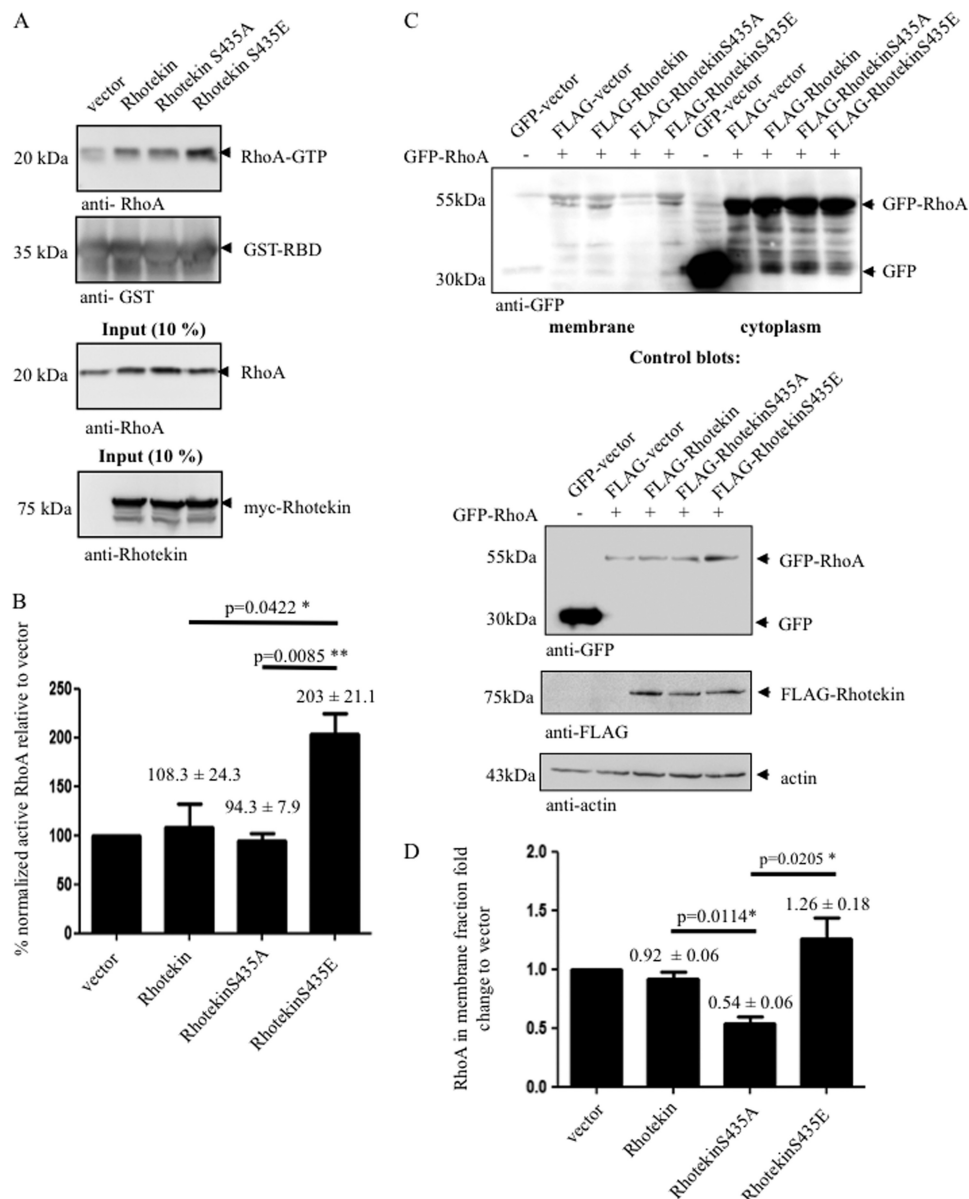
ing the nonphosphorylatable rhotekin mutant (S435A). In marked contrast, expression of the phosphomimetic S435E rhotekin mutant resulted in a significant, about 2-fold increase in active RhoA levels compared with empty vector (Fig. 5, *A* and *B*). The C terminus of RhoA is important for its subcellular localization. RhoA is post-translationally modified by geranylgeranylation at a conserved C-terminal cysteine residue followed by methylation and proteolytic removal of the last three amino acids. Prenylation of RhoA anchors the GTPase in the membrane and is important to control protein stability and cellular protein levels. Correct membrane insertion of RhoA is also crucial for its effect on cellular processes, such as transformation, cell migration, and cytoskeleton organization (29). Thus, we investigated the effects of PKD2-induced rhotekin phosphorylation on RhoA membrane insertion as a potential mechanism of modulating RhoA activity. The different rhotekin phosphosite mutants were transfected together with GFP-RhoA in HEK-293T cells, and subcellular fractionation

assays were performed. Interestingly, RhoA membrane insertion was significantly decreased upon expression of the nonphosphorylatable rhotekin S435A mutant but substantially increased in the presence of the rhotekin S435E mutant (Fig. 5, *C* and *D*), indicating a role of rhotekin phosphorylation in the regulation of RhoA activity by modulating its anchoring in the plasma membrane.

Rhotekin Ser-435 Phosphorylation Enhances Actin Stress Fiber Formation—Rho GTPases can be activated by different mechanisms, and once activated, they can associate with various effectors to regulate diverse cellular processes, including actin polymerization, membrane traffic, microtubule stability, cytokinesis, and cell cycle regulation (30). Activation of RhoA in fibroblasts induces distinct cytoskeletal remodeling. This includes the formation of contractile actin-myosin filaments (stress fibers) and of associated focal adhesion complexes (31). Having observed that the phosphomimetic mutant of rhotekin (S435E) significantly induces RhoA activity, we sought to deter-

FIGURE 5. **PKD phosphorylation of rhotekin at Ser-435 induces RhoA activation.** *A*, lysates of HEK-293T cells transfected with Myc-empty vector, wild-type rhotekin (*Myc-Rhotekin*), or the nonphosphorylatable (*Myc-Rhotekin S435A*) or phosphomimetic rhotekin mutant (*Myc-Rhotekin S435E*) were incubated with GST-RBD immobilized on glutathione-Sepharose beads, and retained GTP bound RhoA was assessed by Western blotting with RhoA antibody. To verify the expression levels of rhotekin wild type and mutants and total RhoA, lysates were subjected to Western blotting with Myc and RhoA antibody, respectively. *B*, quantification of the band intensities of GTP-bound RhoA (active RhoA) bound to GST-RBD was done as described under "Experimental Procedures." Shown are the means ± S.E. (*error bars*) of four independent experiments. *C*, subcellular fractionation of RhoA. HEK-293T cells were transfected with GFP vector, wild type rhotekin, or the phosphosite mutants together with GFP-RhoA and subjected to subcellular fractionation in order to separate membrane and cytoplasm fractions, respectively. Equal amounts of fractions were resolved on 10% SDS gels and subjected to Western blotting. RhoA in membrane and cytoplasm fractions was probed with anti-GFP antibody. *D*, quantitative analysis of GFP-RhoA membrane insertion was performed using densitometry of bands. Shown are relative changes of GFP-RhoA membrane insertion with means and S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

mine the effect of rhotekin Ser-435 phosphorylation on the formation of actin stress fibers. NIH-3T3 cells were transfected with wild-type or mutant rhotekin constructs, and cells were stained with phalloidin to determine the F-actin organization. It has been previously shown that lysophosphatidic acid and cholecystokinin are both potent activators of RhoA and promote actin stress fiber formation (31, 32) by acting on their respective heptahelical receptors. About 40% of cells transfected with wild-type rhotekin exhibited actin stress fibers (Fig. 6, *A* (*top*) and *B*). This can be explained by the fact that overex-

pression of wild-type rhotekin itself slightly increases activity of RhoA above the basal level. About 30% of cells transfected with the not phosphorylatable rhotekin mutant (S435A) exhibited residual actin stress fibers (Fig. 6, *A* (*middle*) and *B*). In marked contrast, 80% of the transfected cells were positive for actin stress fibers upon expression of the phosphomimetic rhotekin mutant (S435E) (Fig. 6, *A* (*bottom*) and *B*), and there were significantly more actin bundles in these cells compared with cells expressing wild type or non phosphorylatable rhotekin. Thus, our data suggest that PKD-mediated phosphorylation of rho-
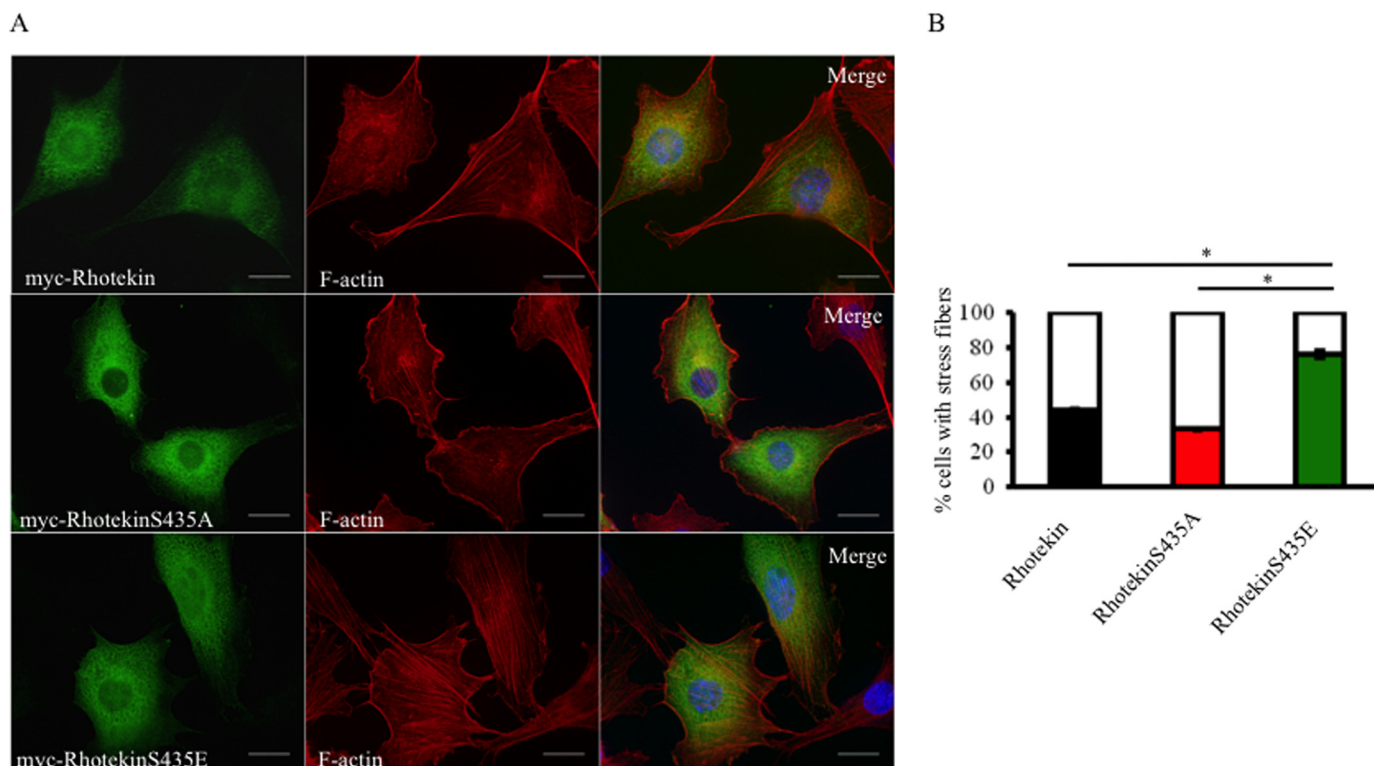
FIGURE 6. **Phosphorylation of rhotekin at Ser-435 enhances stress fiber formation in serum-starved NIH-3T3 cells.** *A*, NIH-3T3 cells transfected with wild-type rhotekin (*myc-Rhotekin*; *top*), dephosphorylatable mutant (*myc-RhotekinS435A*; *middle*), or phosphomimicking mutant (*myc-RhotekinS435E*; *bottom*) were subjected to overnight serum starvation. This was followed by cell fixation and immunostaining with Myc-antibody/Alexa-488 and phalloidin-Alexa-568 to visualize rhotekin and F-actin, respectively. *Scale bars*, 20 μm. *B*, the histogram shows the quantification of the percentage of fibroblast cells positive for stress fibers for at least 100 cells coexpressing wild-type rhotekin (*Rhotekin*), dephosphorylatable mutant (*RhotekinS435A*), or phosphomimicking mutant (*RhotekinS435E*). Shown are the means $\pm$ S.E. (*error bars*) of three independent experiments. *, $p < 0.05$.

tekin at Ser-435 plays an essential role in regulating RhoA activity and thereby modulating actin cytoskeletal remodeling.

DISCUSSION

Members of the PKD family of serine/threonine kinases are prominent downstream targets of the PKC signaling cascade. They are involved in multiple biological processes in which they primarily exert their function by phosphorylating specific target substrates. Due to their important role in both physiological and pathological cellular processes, several potential PKD inhibitors have been developed, some of which may turn out to be useful in a therapeutic setting (33). On the other hand, it is crucial to identify potential PKD substrates that actually mediate these cellular processes. Until now, only a few substrates of PKDs are known, including RIN1, Kidins220, histone deacetylase 5, phosphatidylinositol 4-kinase IIIβ (PI4Kβ), E-cadherin, sphingosine kinase 2, ceramide transfer protein, cAMP-response element-binding protein, Par-1b, Bit-1, cortactin, EVL-1, β-catenin, slingshot phosphatase, and oxysterol-binding protein (8, 9, 34–46). The identification of further PKD substrates will give us a better insight into the mechanisms of PKD-mediated subcellular functions.

Having identified rhotekin as a potential substrate of PKDs in our *in silico* algorithm, we performed the classical *in vitro* kinase assay to experimentally prove that rhotekin is indeed a *bona fide* PKD substrate. Rhotekin was found to be phosphorylated by all three PKD isoforms (Fig. 1). We performed coimmunoprecipitation assays to check whether rhotekin and

PKDs physically associate with each other. However, we did not observe any direct physical association between these proteins (data not shown). This implies that the interaction between PKDs and rhotekin is likely to be a typical transient kinase substrate interaction. A similar result was reported for the PKD substrate PI4Kβ (34). Mass spectrometry identified Ser-435 as the critical site targeted by PKDs, which matched with the Scansite prediction. These data were further verified by generating a Ser(P)-435 antibody that detected the *in vivo* phosphorylation of both overexpressed and endogenous rhotekin in the presence of PKD2 or PKD activating phorbol esters (Figs. 3 and 4). Rhotekin is a scaffold type effector protein for RhoA that binds the small G protein in a GTP-dependent manner. Coimmunoprecipitation assays with constitutively active RhoA-Val-14 did not reveal any difference in binding properties between wild type, phosphomimetic, or nonphosphorylatable rhotekin mutants, demonstrating that Ser-435 phosphorylation has no effect on rhotekin-RhoA interaction (data not shown). Interestingly, rhotekin phosphorylation at Ser-435 enhanced RhoA activity as demonstrated by a RhoA activity assay. Membrane association is critical for the function of Rho family proteins. As a mechanism responsible for the increased RhoA activation, we could establish that PKD2-induced rhotekin phosphorylation substantially increases the membrane anchoring of the RhoA GTPase. *Vice versa*, expression of a nonphosphorylatable rhotekin mutant significantly decreased the RhoA membrane insertion. Thus, PKD2-phosphorylated rho-

tekin confers spatial restriction to RhoA. Active RhoA is known to be a major player in regulating diverse cellular processes, including actin cytoskeletal remodeling. Recently, PKD1 was also shown to be a crucial player in F-actin reorganization and actin polymerization through phosphorylation of slingshot phosphatase and cortactin, respectively (43, 44, 46). Because Ser-435 phosphorylation of rhotekin activates RhoA, we analyzed the effect of the S435E rhotekin mutant on actin stress fiber formation. We found that the percentage of fibroblasts positive for stress fibers was significantly enhanced in the presence of rhotekin S435E mutant.

Previous reports have shown that Rho/Rho kinases act as upstream regulators of PKD1 (47–49), and particularly RhoA signaling has been implicated in regulation of cell motility (44). On the other hand, it is well established that formation of integrin-dependent cell-ECM adhesion and cadherin-dependent cell-cell adhesion as well as PI3K activation affect the function of guanine nucleotide exchange factors and GTPase-activating proteins for Rho family GTPases. These positive and negative regulatory loops appear to be important for cell motility and perhaps other processes (30). Our data suggest that PKD-mediated RhoA activation via rhotekin phosphorylation could be a novel feedback loop regulating the activity of RhoA in the context of actin cyoskeletal rearrangement and cell motility. In conclusion, our data establish a novel mechanism by which PKD2 via phosphorylation of rhotekin affords spatial regulation and modulates the activity of the RhoA GTPase.

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