The Rnd proteins (Rnd1, Rnd2 and Rnd3/RhoE) form a distinct branch of the Rho family of small GTPases. Altered Rnd3 expression causes changes in cytoskeletal organization and cell cycle progression. Rnd3 functions to decrease RhoA activity, but how Rnd3 itself is regulated to cause these changes is still under investigation. Unlike other Rho family proteins, Rnd3 is regulated not by GTP/GDP cycling, but at the level of expression and by post-translational modifications such as phosphorylation and farnesylation.

We show in the present study that, upon PKC (protein kinase C) agonist stimulation, Rnd3 undergoes an electrophoretic mobility shift and its subcellular localization becomes enriched at internal membranes. These changes are blocked by inhibition of conventional PKC isoforms and do not occur in PKCα-null cells or to a non-phosphorylatable mutant of Rnd3. We further show that PKCα directly phosphorylates Rnd3 in an in vitro kinase assay. Additionally, we provide evidence that the phosphorylation status of Rnd3 has a direct effect on its ability to block signalling from the Rho–ROCK (Rho-kinase) pathway. These results identify an additional mechanism of regulation and provide clarification of how Rnd3 modulates Rho signalling to alter cytoskeletal organization.

Key words: GTPase, phosphorylation, plasma membrane, protein kinase C, Rnd3, stress fibre.

INTRODUCTION

Members of the Rho family of small GTPases are involved in the regulation of cell growth and survival, as well as organization of the actin cytoskeleton to control cell shape and cell motility [1]. These proteins act as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound form, the latter of which is then able to interact preferentially with effector molecules. The most thoroughly characterized proteins of this family of small GTPases are RhoA, Rac1 and Cdc42 [2]. Activation of Rho leads to formation of stress fibres and focal adhesions [3], whereas activation of Rac and Cdc42 lead to formation of lamellipodia and filopodia respectively [4-5]. The Rnd family of proteins (Rnd1, Rnd2 and Rnd3/RhoE, also known as Rho6, Rho7 and Rho8 respectively) form a unique branch of the Rho family [6]. One striking difference between Rnd proteins and other members of the Rho family is their ability to effect actin cytoskeleton contraction and for their ability to alter the cytoskeleton [12]. Several small GTPases of the Ras and Rho families have been shown to be substrates for phosphorylation on serine residues at their C-terminal regions immediately upstream of the CAAX motif, and these phosphorylation events have been demonstrated to have functional consequences. We have shown that the previously discovered phosphorylation of the C-terminus of K-Ras4B [13] is directed by PKC (protein kinase C) at Ser41 [14]. This phosphorylation causes K-Ras4B to translocate from the plasma membrane to the mitochondria, resulting in the biological consequence of enhanced apoptosis [14]. We reasoned that the location and function of Rnd3 might also be regulated in a similar manner by phosphorylation of a C-terminal serine residue. We have shown previously that Rnd3 binds to and is a substrate for ROCK1 (Rho-kinase 1), and that this phosphorylation regulates its stability, as
well as its localization [15]. The consensus motifs for ROCK1 and PKC are similar; thus Rnd3 might be a target of both of these kinases.

EXPERIMENTAL

Antibodies and reagents

Antibodies detected HA (haemagglutinin; HA.11 clone 16B12) and Myc (clone 9E11) (Covance); β-actin (clone AC-74), FLAG (M2) and PMA (Sigma); GFP (green fluorescent protein; clone 3E6; Molecular Probes); PKCa (clone 3; BD Biosciences); RhoE (clone 4; Upstate Technologies); phosphosine PKC substrate (Cell Signaling Technology); and total MYPT1 (myosin light chain phosphatase 1) and P-MYPT1 (phosphorylated MYPT1) (Millipore). Anti-Rnd3 serum has been described previously in [16]. Other reagents included ionomycin and Y-27632 (Calbiochem), bryostatin-1 and GÖ-6976 (BIOMOL Research Laboratories) and CIP (calf intestinal phosphatase; New England Biolabs).

Molecular constructs

Rnd3 expression constructs were generated by inserting the full-length human Rnd3 cDNA into the BamHI sites of pCGN-hyg [17] and pEGFP-C1 (Clontech) or into the BamHI and EcoRI sites of pGEX-2T. Rnd3-S240A, Rnd3-SAAX (STVM), Rnd3-S240E and Rnd3-S7,11,240A mutants were generated using the QuickChange Mutagenesis Kit (Stratagene). Full-length WT and KD (kinase-deficient; K368R) rat PKCa cDNA [a gift from William Davis, Department of Cell and Molecular Physiology, UNC-CH (University of North Carolina at Chapel Hill), Chapel Hill, NC, U.S.A.] were PCR amplified and inserted into the XhoI and HindIII sites of both pEGFP-C1 and pCMV-3b to generate GFP–PKCa and Myc–PKCa expression constructs respectively. The FLAG–Rnd3 expression construct was generated by inserting full-length human WT Rnd3 cDNA into the EcoRI and XhoI sites of pHTT-FLAG3 (a gift from Yanping Zhang, Department of Radiation Oncology and Lineberger Comprehensive Cancer Center, UNC-CH). Generation of FLAG–Rnd3–(S7A, S11A, S210A, T214A, S218A, S222A, S240A) (henceforth termed Rnd3–All A) has been described previously [15]. To generate the GFP–Rnd3–All A expression construct, the Rnd3 open reading frame from FLAG–Rnd3–All A was inserted into the HindIII and SalI sites of pEGFP-C3. To generate the GST (glutathione transferase)–Rnd3–All A expression construct, the Rnd3 open reading frame from FLAG–Rnd3–All A was inserted into the EcoRI and XhoI sites of pGEX-4T. All sequences were verified by the Genome Analysis Facility at UNC-CH.

Cell culture and transfections

NIH 3T3 cells were maintained in DMEM-H (high-glucose Dulbecco’s modified Eagle’s medium) (Gibco/Invitrogen) containing 10% FCS (fetal calf serum) (Invitrogen) and P/S (penicillin-strepto-mycin, Invitrogen) at 37°C in a humidified atmosphere of 10% CO2. Isolation of PKCa+/+ and PKCa−/− MEFs (mouse embryonic fibroblasts) has been described previously [18]. These cells were cultured in DMEM-H without sodium pyruvate (Sigma) containing 10% FCS (fetal calf serum), glutamine and P/S (Invitrogen), and maintained at 37°C in a humidified atmosphere of 5% CO2. Expression vectors were transfected into NIH 3T3 cells using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer’s instructions. Expression vectors were transfected into PKCa MEFs using Lipofectamine™ and Lipofectamine™ Plus reagents (Invitrogen) according to the manufacturer’s instructions.

CIP treatment assay

Equal amounts of lysate (devoid of phosphatase inhibitors) from NIH 3T3 cells expressing HA–Rnd3 (treated with or without 100 nM PMA for 10 min) were incubated in phosphatase buffer (100 mM NaCl, 50 mM Tris/HCl, pH 7.9, 10 mM MgCl2 and 1 mM dithiothreitol) with or without 20 units of CIP at 37°C for 1 h. Lysates were resolved on SDS/PAGE (12% gels), transferred on to Immobilon PVDF (Millipore) and blotted with anti-HA antibody.

Live cell imaging

To visualize the effects of PKC activation on Rnd3 localization in real time, NIH 3T3 cells were transiently transfected with GFP–Rnd3. After 24 h, cells were treated with either bryostatin-1 (100 nM) or PMA (100 nM) and ionomycin (500 µg/ml). Live cell images were captured on a Zeiss 510 LSM confocal microscope at ×20 magnification and analysed using LSM 5 Image Browser software (Zeiss). To evaluate a role for different PKC isoforms in modulating Rnd3 localization, NIH 3T3 cells were transiently transfected with GFP–Rnd3 as above. After 24 h, cells were incubated with either DMSO vehicle or the PKC inhibitor Gö-6976 (2.5 µM). After 3 h, cells were treated with PKC agonists PMA (100 nM) and ionomycin (500 µg/ml), and live cell images were captured by confocal microscopy at 5 min intervals as described above.

In vitro kinase assay

GST alone, GST–Rnd3–WT and GST–Rnd3–All A were purified using glutathione–Sepharose beads. His-tagged vinculin tail (aa 881–1135) was a gift from Sharon Campbell (Department of Radiation Oncology and Biophysics, UNC-CH). Purified proteins were incubated with or without recombinant PKCa in kinase buffer supplemented with PKC lipid activator (Upstate Technologies) in the presence of 32P-labelled phosphate. The kinase reaction was incubated at 40°C for 30 min and the reactions were stopped by addition of 2× Laemmli sample buffer. Samples were resolved by SDS/PAGE, stained with Coomassie Blue and developed by autoradiography.

Western blot analysis

Cells were washed with PBS, lysed in RIPA lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS and 0.5% sodium deoxycholate and supplemented with Complete Protease Inhibitor Cocktail tablets (Roche) plus PMSF and sodium pervanadate) and centrifuged to remove insoluble material. Then 2× Laemmli sample buffer was added to equivalent amounts of cellular lysates which were then resolved on SDS/PAGE (12% gel) and transferred on to Immobilon PVDF membranes. Membranes were blocked in 5% (w/v) non-fat dried skimmed milk powder in TBS-Tween 20 (20 mM Tris/HCl, pH 7.6, 137 mM NaCl and 0.2% Tween 20) and probed with appropriate primary antibodies followed by anti-mouse or rabbit IgG–horseradish-peroxidase-conjugated secondary antibody (Amersham Biosciences). Membranes were then incubated in SuperSignal West Dura Extended Duration substrate (Pierce) and the signal developed on HyBlot CL autoradiography film (Denville Scientific).
RESULTS

Rnd3 subcellular localization is altered upon PKC activation

Inspection of Rnd protein sequences revealed a consensus PKC site at Ser<sup>240</sup> in the C-terminal hypervariable membrane-targeting domain immediately upstream of the CAAX prenylation motif, similar to the arrangement seen in the C-terminus of K-Ras4B. We previously found that this site can be phosphorylated by ROCK1 [15], which shares a similar consensus sequence to that of PKC. We reasoned that the subcellular localization of Rnd3 might also be regulated by a combination of prenylation and C-terminal phosphorylation. We therefore decided to use PKC agonists to test the effects of PKC activation on Rnd3 subcellular localization. To visualize these effects in living cells, we treated NIH 3T3 mouse fibroblast cells transiently expressing GFP-tagged Rnd3 with the non-phorbol PKC-specific agonist bryostatin-1. Live cell images were taken before and 10 min after treatment. As shown in Figure 1(A), treatment with bryostatin-1 caused rapid loss of Rnd3 from the plasma membrane and enrichment in the cytosol and internal membranes. To determine whether this response was unique to bryostatin-1 or a reproducible consequence of activating PKC, we also treated cells with the phorbol ester PMA and the calcium ionophore ionomycin. NIH 3T3 cells expressing GFP–Rnd3 were treated with PMA plus ionomycin, and live cell images were taken at 5 min increments. As shown in Figure 1(B), treatment with PMA plus ionomycin, similar to bryostatin-1, also caused the loss of Rnd3 from the plasma membrane and enrichment in the cytosol and internal membranes, as we reported previously [15] for PMA treatment of HeLa cells. The change in Rnd3 subcellular localization after treatment with distinct types of PKC agonists indicates that PKC activity is inversely correlated with Rnd3 binding to the plasma membrane, and suggests further that activated PKC may cause phosphorylation of Rnd3 itself and that the phosphorylation state of Rnd3 has direct consequences on its cellular localization. To begin to elucidate the identity of the specific PKC family member(s) responsible for the change in Rnd3 subcellular localization after stimulation with broad-based PKC activators, we employed G<sub>6</sub>-6976, which selectively inhibits conventional but not novel PKCs [19]. NIH 3T3 cells expressing GFP–Rnd3 as above were treated with either G<sub>6</sub>-6976 or DMSO vehicle alone for 3 h prior to stimulation with PMA plus ionomycin. As shown in Figure 1(C), G<sub>6</sub>-6976, but not the vehicle control, blocked alterations in Rnd3 localization. This result indicates that at least one conventional PKC isoform is involved in the regulation of the subcellular location of Rnd3. Because NIH 3T3 cells express only the <i>α</i> isoform of conventional PKCs [20], it is likely that PKC<sub>α</sub> is the major isoform responsible for the effects seen with Rnd3.

Rnd3 is phosphorylated upon PKC activation

To evaluate the possible direct involvement of Rnd3 phosphorylation in modulating its location, we wished to determine whether Rnd3 itself becomes phosphorylated upon activation of PKC. NIH 3T3 cells transiently expressing HA-tagged Rnd3 were treated with PKC agonists and inhibitors as in Figure 1(C). Cell lysates were collected, resolved by SDS/PAGE and immunoblotted with anti-HA antibody. As shown in Figure 2(A), a slightly slower-migrating band consistent with post-translationally modified HA–Rnd3 appeared in lysates of cells stimulated with PMA, but not with DMSO vehicle. This result is consistent with phosphorylation of Rnd3 upon PKC activation. Furthermore, G<sub>6</sub>-6976 blocked this mobility shift, suggesting that it is mediated by a conventional PKC. Rnd3 can also be a subject of ROCK-mediated phosphorylation [15,21]. ROCK and conventional PKCs share a common phosphorylation recognition sequence [22] and these kinases can directly phosphorylate identical residues [23]. We therefore investigated whether ROCK-mediated Rnd3 phosphorylation was stimulated by treatment with PMA. As seen in Figure 2(A), pretreatment of NIH 3T3 cells with the ROCK-selective inhibitor Y-27632 failed to prevent the mobility shift of HA-tagged Rnd3 when cells were treated with PMA, although it did block ROCK activation as shown by abrogation of P-MYPT1 (results not shown). Next, to confirm that the slower-migrating band represents a phosphorylation event, we
lysates were resolved by SDS/PAGE. HA-tagged WT Rnd3 and a SAAX mutant were treated with PMA (100 nM) for 10 min and cell lysates were resolved by SDS/PAGE and blotted with anti-HA antibody. With PMA (100 nM) plus ionomycin (500 nM) or Go-6976. We also targeted by PKC. To determine whether the S240A mutation rendered Rnd3 PKC-insensitive, we evaluated whether it retained or lost the PKC-induced mobility shift on SDS/PAGE. Contrary to our initial hypothesis, but consistent with the cell morphology data, both the WT and the S240A mutant forms of Rnd3 displayed the same mobility on SDS/PAGE in the absence or presence of PKC activation (Figure 3A). Taken together, we concluded that phosphorylation of Rnd3 at Ser240 alone is not sufficient to regulate Rnd3 subcellular localization or to produce the mobility-shifted form of Rnd3. These results are consistent with our observations that the mobility shift is induced by phosphorylation of other residues, as indicated below.

Additional sites of PKC-mediated phosphorylation in Rnd3

In optimizing the SDS/PAGE gel mobility-shift experiments using HA-tagged Rnd3, we employed a rabbit antiserum produced against the N-terminus of Rnd3 [16]. Surprisingly, this antiserum did not detect the mobility shift of HA–Rnd3 in lysates from NIH 3T3 cells that had been treated with PMA. Yet this shift was seen reproducibly when immunoblotting with either an antibody directed against the HA epitope tag or a mouse monoclonal antibody whose immunogen was the entire Rnd3/RhoE protein (Figure 3B). We therefore postulated that the site or sites of phosphorylation responsible for the shifted form of Rnd3 must be located in the first 15 amino acids that were used in producing the Rnd3 rabbit antiserum. Visual inspection of the Rnd3 sequence, and the phosphorylation prediction program NetPhos2.0, revealed two additional consensus PKC phosphorylation sites at Ser2 and Ser11, previously shown to be sites for phosphorylation by ROCK1 [15], which has a similar consensus motif. Together with the previous data demonstrating a correlation between the alteration of the subcellular localization of Rnd3 and the presence of a mobility-shifted form of Rnd3 on SDS/PAGE, we hypothesized that phosphorylation of Rnd3 within this unique N-terminal extension, which would introduce negative charges, could be responsible for the effects on Rnd3 seen after treatment with PKC agonist. We reasoned that phosphorylation of Rnd3 at Ser2 and
Ser\textsuperscript{11} in the N-terminal extension, along with phosphorylation at Ser\textsuperscript{240}, may disrupt the polar interactions of Rnd3 with the plasma membrane. Site-directed mutagenesis was then used to generate GFP-tagged versions of Rnd3 that contained alanine substitutions at Ser\textsuperscript{7} and Ser\textsuperscript{11}, along with Ser\textsuperscript{240}, and this mutant GFP–Rnd3 construct was expressed transiently in NIH 3T3 cells. However, as with GFP–Rnd3-S240A, the subcellular localization of the triple serine mutant, GFP–Rnd3-S7,11,240A, was also indistinguishable from that of GFP–Rnd3-WT after treatment with PMA (Figure 3C). One possible explanation for lack of effect of the triple mutant S7A/S11A/S240A is that phosphorylation at one or more other site(s) is the primary target of PKC. To explore this possibility, six serine residues and a threonine residue (Ser\textsuperscript{7}, Ser\textsuperscript{11}, Ser\textsuperscript{210}, Thr\textsuperscript{214}, Ser\textsuperscript{220}, Ser\textsuperscript{222} and Ser\textsuperscript{240}) in Rnd3 were mutated to the corresponding phospho-deficient alamines to generate a non-phosphorylatable form of Rnd3 (Rnd3-All A) [15] and this non-phosphorylatable mutant was evaluated in a PMA-treatment translocation assay. As seen in Figure 4(A), stimulation of NIH 3T3 cells with PMA caused the loss of GFP-tagged Rnd3-WT from the plasma membrane. Consistent with a requirement for Rnd3 to become phosphorylated in order for it to translocate upon PKC activation, PMA stimulation did not cause the loss of the non-phosphorylatable Rnd3-All A mutant from the plasma membrane. Furthermore, a FLAG-tagged version of Rnd3-All A did not display a gel mobility shift when expressed in NIH 3T3 cells stimulated with PMA (Figure 4B), as was seen with FLAG–Rnd3-WT. Our extensive efforts to identify the relevant specific Rnd3 phosphorylation site(s) through proteomic means have been unsuccessful. Thus, although further work will be needed to identify the minimal number of PKC phosphorylation sites needed for membrane translocation, it is clear that both the mobility shift and the translocation seen upon PKC activation require that Rnd3 itself is able to become phosphorylated.

PKC\(\alpha\) is the isoform responsible for Rnd3 phosphorylation

Although the specific target residue(s) have not yet been determined, the exact identity of the PKC isoform(s) responsible for the phosphorylation and altered localization of Rnd3 upon PKC activation also remained to be uncovered. On the basis of the PKC inhibitor data shown earlier (Figures 1C and 2A) and the fact that NIH 3T3 cells express only the \(\alpha\) isoform of conventional PKCs [20], we hypothesized that PKC\(\alpha\) was the
isoform involved. Therefore, we performed additional studies in MEFs in which PKCα had been genetically ablated [18]. We first tested whether PKCα is required for the electrophoretic mobility shift of Rnd3 seen upon stimulation with PKC agonists. PKCα−/− MEFs and WT control cells transiently expressing HA–Rnd3 were treated with the PKC agonist PMA. Lysates from these cells were resolved on SDS/PAGE and immunoblotted with anti-HA antibody. Furthermore, lysates were blotted with an anti-α-actin antibody, demonstrating the absence of the PKCα protein in PKCα−/− MEFs (Figure 5A). The slower-migrating band of Rnd3 was seen only in the WT MEF cells and not in the PKCα−/− MEF cells, demonstrating that the PKCα isoform is required for the mobility-shifted form of Rnd3 which we showed previously represents PKC-mediated phosphorylated form of Rnd3.

To confirm that the kinase activity of PKCα is required for its effects on Rnd3 phosphorylation, we re-introduced either WT or KD PKCα-K368R into PKCα−/− MEFs and looked for restoration of the appearance of the slower-migrating form. The PKCα-K368R mutant is considered KD as it abolishes ATP-binding ability. PKCα−/− MEFs, transiently expressing HA–Rnd3 along with empty Myc vector only, Myc–PKCα-WT or Myc–PKCα-KD were treated with the PKC agonist PMA, and cell lysates were resolved on SDS/PAGE and immunoblotted with anti-HA antibody. As shown in Figure 5(B), a gel mobility shift of HA–Rnd3 was seen only upon re-introduction of PKCα-WT, but not KD PKCα. Thus the kinase activity of PKCα is required for Rnd3 phosphorylation. Next, we transiently expressed GFP vector only and GFP–Rnd3 in both PKCα−/− and matched control WT MEFS. Cells were treated with the PKC agonist PMA and images were taken before and after treatment. As expected, PMA treatment had no effect on the cellular localization of GFP alone in either PKCα−/− or the matched control WT MEFS (Figure 5C). Consistent with a requirement for the PKCα isoform, PMA treatment caused the loss of GFP–Rnd3 from the plasma membrane in WT MEFS but not in PKCα−/− MEFS.

Finally, an in vitro kinase assay was used to determine whether PKCα directly phosphorylates Rnd3. As seen in Figure 5(D), GST–Rnd3-WT, but neither the GST tag alone nor GST–Rnd3–All A, became phosphorylated in the presence of recombinant PKCα. As a positive control, a truncated tail of vinculin (aa 881–1135) was also phosphorylated in the presence of PKCα. Furthermore, kinase activity was visualized by the presence of autophosphorylated PKCα. A lack of incorporated 32P in the Rnd3–All A mutant suggests that one or more of the Ser/Thr residues mutated in this construct is phosphorylated by PKCα. Thus the data presented, through the use of a conventional PKC-specific inhibitor and PKCα−/− MEFS, along with the direct phosphorylation of Rnd3 by PKCα, give strong evidence that PKCα is likely to be the kinase responsible for Rnd3 phosphorylation in the present study.

PKCα-dependent Rnd3 phosphorylation down-regulates Rnd3 inhibitory activity and leads to increased signalling through the Rho–ROCK pathway

As mentioned earlier, Rnd3 exerts its biological activity by counteracting the effects of RhoA signalling. Because we predicted that PKC-mediated phosphorylation decreases Rnd3 activity, we investigated whether Rnd3 phosphorylation leads to an increase in signalling through the Rho–ROCK pathway. To this end, NIH 3T3 cells were transiently transfected with the GFP only, GFP–Rnd3-WT or GFP–Rnd3–All A expression constructs. Transfected cells were treated with either DMSO vehicle or PMA and then fixed and stained with rhodamine-conjugated phalloidin to mark actin. As seen in Figure 6(A), PMA treatment caused not only translocation of GFP–Rnd3 from the plasma membrane but also the restoration of stress fibres, along with greater spreading and a flattened appearance of the cells. None of these changes were seen when cells were treated with only DMSO vehicle. In direct opposition to the results seen with GFP–Rnd3–WT, PMA treatment had no effect on the plasma membrane localization of the PKC-insensitive mutant GFP–Rnd3–All A; stress fibres were not restored, and the cells did not flatten and spread. To uncover a possible molecular mechanism for the restoration of stress fibres and cell spreading in PMA-treated cells expressing Rnd3–WT but not Rnd3–All A, lysates from PMA-treated cells were resolved on SDS/PAGE and blotted for P-MYPT1, a downstream target of the Rho–ROCK pathway [24].
Figure 5  PKCα is the isozyme responsible for Rnd3 phosphorylation

(A) PMA stimulation causes a gel mobility shift of Rnd3 in WT MEF cells, but not in PKCα knock-out MEFs. PKCα knock-out MEFs and matched WT MEFs transiently expressing HA–Rnd3 were treated with the PKC agonist PMA (100 nM) for 10 min. Cell lysates were resolved on SDS/PAGE and blotted with anti-HA antibody. Cell lysates were further probed with an anti-PKCα antibody to confirm absence of PKCα protein expression in knock-out MEF cells. The slower-migrating band of Rnd3 was seen only in WT MEF cells (arrow). (B) Re-introduction of PKCα-WT, but not of PKCα dominant-negative (DN), into PKCα knock-out MEFs causes a mobility shift of Rnd3 when cells are treated with PKC agonist PMA. PKCα knock-out MEFs transiently expressing HA–Rnd3 along with either pCMV-vector (v.o.), Myc–PKCα-WT or Myc–PKCα dominant-negative were treated with the PKC agonist PMA (100 nM) for 10 min. Cell lysates were resolved on SDS/PAGE and probed with anti-HA antibody. A gel mobility shift of Rnd3 (arrow) was seen only in lysates from PKCα knock-out cells when WT PKCα was reintroduced. (C) GFP–Rnd3 translocates from the plasma membrane in the WT MEF cells (+/+) but not PKCα knock-out MEFs (−/−), after PKC stimulation. WT MEFs and PKCα knock-out MEFs were transiently transfected with either GFP vector or GFP–Rnd3. MEFs were treated with the PKC agonist PMA (100 nM) for 10 min and live cell images were taken. Scale bar, 20 μm. (D) Rnd3 is phosphorylated by PKCα in vitro. GST alone, GST–Rnd3-WT, GST–Rnd3-All A and a tail fragment of vinculin (aa 881–1135) were used as substrates in a PKCα in vitro kinase assay. Rnd3-WT, but not Rnd3-All A, incorporated 32P-labelled phosphate. *Autophosphorylated PKCα.

As seen in Figure 6(B), in Rnd3-WT-expressing cells, the levels of P-MYPT1 were higher after treatment with PMA, as compared with treatment with DMSO vehicle only. In contrast, Rnd3-All A effectively acted as a dominant-negative in this pathway, abrogating the ability of PKC activation to increase P-MYPT1. The data are thus consistent with a model in which Rnd3 located at the plasma membrane is able to disrupt signals from the Rho–ROCK pathway that are involved in stress fibre formation/maintenance. We envision that, when Rnd3 becomes phosphorylated after PKC activation and is translocated from the plasma membrane, it is no longer able to disrupt the Rho–ROCK signalling pathway. The Rnd3-All A mutant, which is no longer subject to PKC-mediated phosphorylation, remains located on the plasma membrane, even after PKC activation. Hence, in Rnd3-All A-expressing cells, signals coming from the Rho–ROCK pathway can still be disrupted by this PKC-insensitive mutant Rnd3. Previous work has demonstrated that the Rnd3-All A mutant is still able to interact with ROCK, as demonstrated by in vitro binding and pull-down assays [15]. These results demonstrate that PKCα phosphorylation of Rnd3 represents an important negative-feedback loop that may be critical to restoration of signalling through the Rho–ROCK pathway following transient activation of the GTPase-insensitive Rho family protein Rnd3.

DISCUSSION

It has long been appreciated that Rnd3/RhoE is constitutively GTP-bound [9], and therefore that its activity must be regulated by means other than GTP–GDP cycling. Rnd3 protein expression
by other means might be required to regulate dynamic signalling processes. Rnd3/RhoE is known to interact with and be a direct target of the serine/threonine kinase ROCK1 [15,21]. In the present study we have presented evidence that post-translational regulation of Rnd3 activity can also be accomplished via differential subcellular localization due to PKCα-mediated phosphorylation. These results add an additional mechanism of regulation to those documented previously, and provide clarification of how Rnd3 modulates Rho signalling to alter cytoskeletal organization. Several closely related proteins have been shown previously to be substrates for phosphorylation by PKC. In particular, K-Ras4B was shown to be a substrate for PKC phosphorylation in its C-terminal polybasic region [13], and we have shown that this phosphorylation influences both its subcellular localization and function [14]. The presence of similar serine residues in Rnd3 at sites homologous with those of phosphorylation in related proteins prompted us to investigate whether Rnd3 displayed a similar mode of regulation. Inspection of the Rnd3 amino acid sequence revealed a potential PKC phosphorylation site at Ser240, just upstream of the CAAX motif in the C-terminal hypervariable membrane-targeting domain.

In the present study we demonstrated that Rnd3 is phosphorylated upon PKC activation, and that inhibition of conventional PKC isoforms abrogates this phosphorylation. However, we have determined that phosphorylation at Ser240 alone could not be responsible for the effects seen with Rnd3 due to PKC activation. Rather, multiple residues found in both the unique N-terminal and C-terminal extensions are necessary. Perhaps phosphorylation of residues located in both the N- and C-terminal extensions would reduce Rnd3 plasma membrane affinity. The exact phosphorylation sites contained within these extensions necessary for loss of Rnd3 from the plasma membrane and translocation to the cytosol still remain to be deciphered. We concluded that phosphorylation of Rnd3 at Ser240 alone is not sufficient to regulate Rnd3 subcellular localization or to produce the mobility-shifted form of Rnd3.

Given that the consensus sequences for PKC and ROCK1 are similar, several of the same Ser/Thr residues in Rnd3 could be phosphorylated by either kinase or by both, depending on the stimulus, as well as the relative abundance of PKC and ROCK1. There is a relative abundance of these proteins in HeLa cancer cells used in our previous study [15] compared with the NIH 3T3 fibroblast cells used here is not known, but cell type differences may be important in phosphorylation of Rnd3 following PMA stimulation. It is also possible that PKC might act upstream of ROCK1, enhancing its ability to interact with and phosphorylate Rnd3. In this regard, we saw only partial inhibition of PKCα-mediated phosphorylation of Ser240 by ROCK inhibitors in HeLa cells [15], and thus it is likely that there is both a direct phosphorylation of at least this site by PKC, as well as an indirect effect via ROCK1-mediated phosphorylation of Ser240, i.e., as a result of an indirect pathway of PKC–ROCK1. However, it is important to note that, regardless of the relative involvement of PKC compared with ROCK, in both of our studies ([15] and the present study) we have observed the same functional consequence of PMA stimulation to induce a shift of Rnd3 from plasma membrane to cytoplasm.

Thus we have also shown in the present study that the phosphorylation state of Rnd3 has direct consequences on its cellular location, with phosphorylation causing loss of plasma membrane localization and translocation to the cytosol. Similar results have been documented for other proteins, such as the ARF (ADP-ribosylation factor) nucleotide-exchange factor ARNO (ARF nucleotide-binding-site opener) and the small GTPase RaIA, where phosphorylation events also result in loss of protein localization.
cytoskeleton. We offer compelling evidence that Rnd3 may
Rho–ROCK pathway, thus leading to remodelling of the actin
cytoskeleton. We suggest that phosphorylation of Rnd3 leads to relocalization
away from sites where it can antagonize signalling from the
Rho–ROCK pathway and the myriad of cell responses they control
coregistration through cytoskeletal organization via actomyosin contractility.

AUTHOR CONTRIBUTION
James Madigan designed the research, performed experiments and analysed the data, and
wrote the manuscript. Brian Bodemann designed the research, performed experi-
ments and analysed data. Donita Brady, Brian Dewar and Patricia Keller performed
experiments and analysed data. Michael Leitges provided critical reagents. Mark Phillips
was involved in research design. Anne Ridley provided critical reagents, suggestions for
research design and manuscript revisions. Channing Der provided funding resources and
project guidance. Adrienne Cox designed the research, analysed the data, provided
funding resources and co-wrote the manuscript.

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