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Cdc42 is involved in PKCε- and δ-induced neurite outgrowth and stress fibre dismantling ☆

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Abstract

We have shown that protein kinase C (PKC)ε, independently of the catalytic domain, induces outgrowth of cellular processes via its regulatory domain in both neural cells and fibroblasts. This was accompanied by stress fibre loss. Here, we have examined the role of the small GTPases, Rac1, and Cdc42, in these PKC-mediated morphological and cytoskeletal changes. Both constitutively active and dominant negative Rac1 and Cdc42 attenuated the PKC-mediated outgrowth of processes. The suppression was larger for Cdc42 than for Rac1. The PKC-mediated dismantling of the stress fibres in both HiB5 and fibroblasts was inhibited by the expression of the Cdc42 mutants whereas they had smaller effects on the stress fibre dismantling induced by the ROCK inhibitor, Y-27632, indicating a more crucial role for Cdc42 in the PKC-mediated pathway. We conclude that Cdc42 is an important downstream factor in the pathway through which PKC mediates morphological and cytoskeletal effects.

Keywords: Protein kinase C; Neurite outgrowth; Stress fibres; Neuroblastoma cells; Fibroblasts; Cdc42; Rac1; ROCK

Neurite outgrowth is an essential process in the developing nervous system leading to the formation of an intricate network of axons and dendrites. However, the capacity to extend long cellular processes is not unique to neuronal cells. Several developmental functions seem to involve the outgrowth and elongation of cellular extensions and it has also been shown to precede cellular migration [1]. Thus, this is a general cell biological process that is of importance for many cellular functions.

The extension of neurites and other cellular processes is caused by dynamic changes in the cytoskeleton which can be regulated by a wide range of signal transduction proteins. One group of proteins that influence many aspects of the cytoskeleton is the family of protein kinase C (PKC) isoforms [2]. This family of proteins can be subgrouped in classical (α, βI, βII, γ), novel (δ, ε, η, θ), and atypical (ι/λ, ζ) isoforms [3]. Particularly the novel isoforms PKCδ and ε have been suggested to positively influence neurite outgrowth [4–8].

We have previously shown that overexpression of PKCε induces neurite outgrowth in neural cells via its regulatory domain and independently of its catalytic activity [9,10]. The effect is limited to the morphological changes and does not involve increased expression of neuronal differentiation [9]. The same effect can be obtained in immortalized hippocampal neural precursor cells, HiB5 cells [11], and in fibroblasts [12] implying that a general cytoskeletal regulatory mechanism is targeted by PKCε. In the HiB5 cells, also PKCδ is neuritogenic but only after the addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The initiation of neurite outgrowth is accompanied by a loss of stress fibres [11]. As for neurite outgrowth, the PKC-induced stress fibre disassembly is independent of the PKC catalytic activity suggesting that they are mediated via a common mechanism and, in addition, both events are counteracted by an active RhoA pathway [11].

☆ *Abbreviations:* EGFP, enhanced green fluorescent protein; FL, full-length; RD, regulatory domain; RA, retinoic acid; NGF, nerve growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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The RhoGTPase family constitutes a group of proteins that are central regulators of the dynamic changes of the actin cytoskeleton morphology that drives the outgrowth of neurites. The Rho GTPases act as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. Two classes of proteins regulate the switch between the two states. Guanine nucleotide exchange factors (GEFs), which activate the Rho GTPase by facilitating the replacement of GDP to GTP, and GTPase activating proteins (GAPs), which inactivate the Rho GTPase by enhancing the intrinsic hydrolytic activity [13].

The Rho family can be subgrouped in three major classes, exemplified by Rac1, Cdc42, and RhoA which in fibroblasts have been shown to induce the formation of lamellipodia, filopodia and stress fibres, respectively [14]. Studies in neuronal cell lines have shown that the Rho family is essential for the development of neurites [15]. The Rac1- and Cdc42-induced lamellipodia and filopodia promote growth cone advance, while RhoA in many cell systems triggers a collapse of the growth cone and the retraction of neurites [16,17].

The aim of this study was to further investigate the mechanisms by which PKC mediates its morphological effects and whether the Rho GTPases, Rac1 and Cdc42, are involved downstream of PKC.

Materials and methods

Plasmids. Expression vectors encoding PKC domains fused to the enhanced green fluorescent protein (EGFP) have previously been described [9]. pRK5 expression vectors encoding myc-tagged Rac1 and Cdc42 were kindly provided by Dr. A. Hall.

Cell culture. Human neuroblastoma SH-SY5Y/TrkA [18], kindly provided by Dr. S. Påhlman, and SK-N-BE(2) cells were maintained in Minimal Essential Medium. Mouse NIH 3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium with sodium pyruvate and 1000 mg/l glucose. The immortalized hippocampal HiB5 cells [19], kindly provided by Dr. R.D.G. McKay, were grown at 33 °C in Dulbecco's modified Eagle's medium without sodium pyruvate and with 4500 mg/l glucose. All mediums were supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cell culture solutions were from Gibco-BRL.

For morphology studies, SH-SY5Y/TrkA cells were trypsinised and seeded at a density of 150,000 cells per 35-mm cell culture dish on glass coverslips in serum-free medium. After 20 min, the medium was changed to medium containing serum and antibiotics. SK-N-BE(2), NIH 3T3 and HiB5 cells were seeded on glass coverslips in regular growth medium at a density of 50,000, 70,000, and 100,000 cells, respectively, per dish.

When indicated, TPA (Sigma) was used at a concentration of 16 nM. The PKC inhibitor GF109203X (Calbiochem) was used at a concentration of 2 μM and the ROCK inhibitor Y-27632 (Calbiochem) was used at a concentration of 10 μM. For differentiation studies, SH-SY5Y/TrkA cells were treated with 100 ng/ml nerve growth factor (NGF; Promega) and SK-N-BE(2) cells were treated with 10 μM retinoic acid (RA; Sigma).

Transfection. Transfections were initiated 24 h after seeding and were done in serum-free medium. For 35-mm dishes, 2 μg DNA and 4 μl Lipofectamine (Gibco-BRL) in 1 ml medium were used and for 100-mm dishes, 6 μg DNA and 12 μl Lipofectamine in 3 ml medium were used. The ratio between the plasmids, pEGFP-N1 (Clontech), and pRK5 with inserts, was in the co-transfection studies in HiB5 and NIH 3T3 cells 1:5 and in the SH-SY5Y/TrkA and SK-N-BE(2) cells equal amounts of plasmids were used. The ratio between the plasmids, pEGFP-N1, and pcDNA4/myc-His (Invitrogen) with inserts, was 1:3 in HiB5 cells.

Immunofluorescence. After transfection, cells were incubated for 16 h before fixation in 4% paraformaldehyde in PBS for 4 min. For detection of F-actin, cells were incubated for 20 min with Alexa Fluor 546-conjugated phalloidin (Molecular Probes) diluted 1:100 in TBS with 0.3% Triton X-100. For detection of myc-tagged Rho GTPases, cells were permeabilised and blocked with 5% normal donkey serum and 0.3% Triton X-100 in TBS for 30 min and thereafter incubated for 1 h with primary mouse anti-myc antibody (Oncogene) diluted 1:50 in TBS. Following washes, cells were incubated for 1 h with secondary TRITC-conjugated donkey-anti-mouse IgG (Jackson ImmunoResearch) diluted 1:200 in TBS, followed by extensive washes. Coverslips were mounted on object slides using 20 μl PVA-DABCO (9.6% polyvinyl alcohol, 24% glycerol, and 2.5% 1,4-diazabicyclo[2.2.2]octane in 67 mM Tris–HCl, pH 8.0).

Morphology studies. Transfected cells were examined with fluorescence microscopy and for quantification of cells with neurites or stress fibres. Two hundred (neurites) or 100 (stress fibres) cells were counted in each experiment. Cells were considered to have long processes if the length of the process was more than the length of two cell bodies and classified as stress fibre positive if they contained two or more fibres spanning the cell body. Digital images were captured with a Sony DKC 5000 camera system.

Western blot analysis. Cells were washed twice in PBS and lysed in RIPA buffer (10 mM Tris–HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 160 mM NaCl, 0.1% sodium dodecyl sulphate, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, and complete protease inhibitor cocktail [Roche]) for 10 min on ice. The lysate was clarified by centrifugation at 18,000*g* for 10 min at 4 °C. Proteins were electrophoretically separated with SDS–PAGE and transferred to a PVDF membrane (Millipore). The membrane was immunoblotted with monoclonal antibodies toward the myc epitope (Oncogene) and thereafter incubated with horseradish peroxidase-linked secondary antibodies (Amersham). Detection was done with the SuperSignal system (Pierce) as substrate and the chemiluminescence was detected with a CCD camera (Fujifilm).

Results

Rac1 and Cdc42 are important for PKC-induced neurite outgrowth in neural cells

The importance of the Rho family members Rac1 and Cdc42 in neurite outgrowth led us to examine whether they influence the PKC-mediated neurite formation previously reported by our group [9–11]. Neurite outgrowth was induced by overexpressing full-length PKCε (PKCεFL) or the regulatory domain (PKCεRD) of PKCε in SK-N-BE(2) neuroblastoma cells. The effect of co-expressing constitutively active, L63Rac1 or L63Cdc42, or dominant negative, N17Rac1 or N17Cdc42, mutants on the neurite outgrowth was investigated (Fig. 1A–C). The expression of the myc-tagged GTPases was detected with immunofluorescent staining of the myc epitope and cells positive for both EGFP and myc were scored. Neither of the Rac or Cdc42 mutants were able to induce neurites on its own (Fig. 1A). As previously reported [9,10], overexpression of PKCεFL-induced neurites in ~40% of the transfected cells (Fig. 1B) while the isolated regulatory domain was more potent, inducing neurite outgrowth in ~60% of the transfected cells (Fig. 1C). Co-expression of constitutively active Rac1 or Cdc42 completely blocked the induction

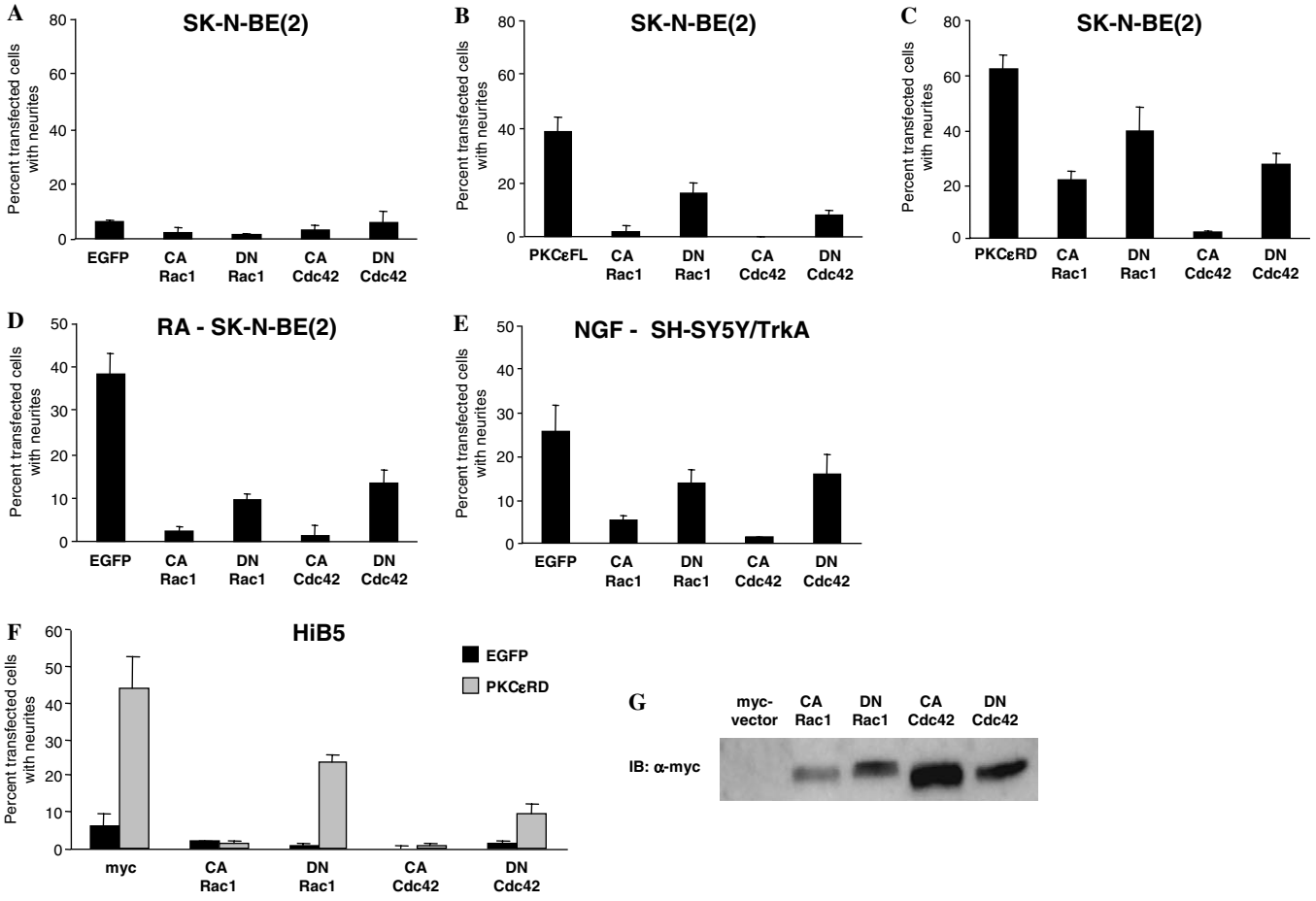

Fig. 1. Constitutively active and dominant negative Rac1 and Cdc42 inhibit neurite outgrowth in neural cells. SK-N-BE(2) neuroblastoma cells were co-transfected with a vector encoding myc-tagged constitutively active or dominant negative Rac1 or Cdc42 together with (A) an empty EGFP-vector, (B) a vector encoding full-length PKCε (PKCεFL) fused to EGFP or (C) a vector encoding the regulatory domain of PKCε (PKCεRD) fused to EGFP. (D,E) Neuroblastoma cells were co-transfected with an empty EGFP-vector together with a vector encoding myc-tagged Rac1 or Cdc42 mutants. After transfection, cells were induced to differentiate by three days of treatment with (D) 10 μM RA in SK-N-BE(2) cells and (E) 100 ng/ml NGF in SH-SY5Y/TrkA cells. (F) HiB5 cells were co-transfected with either empty EGFP-vector or a vector encoding PKCεRD together with either an empty myc-vector or a vector encoding myc-tagged Rac1 or Cdc42 mutants. Sixteen hours (A–C and F) or three days (D,E) after transfection, cells were fixed, stained with antibodies towards myc (A–E), and mounted on object slides. Transfected cells were scored for the presence of neurites longer than two cell bodies. Data (means ± SEM, $n = 3$) are percentage of transfected cells with neurites. (G) HiB5 cells were transfected with either an empty myc-vector or a vector encoding myc-tagged Rac1 or Cdc42 mutants. Sixteen hours after transfection cells, were lysed and analysed with Western blot using antibodies toward the myc-tag.

of neurites in cells expressing PKCεFL. Dominant negative Rac1 or Cdc42 suppressed the neurite induction, with dominant negative Cdc42 having the largest effect (Fig. 1B). PKCεRD-induced neurite outgrowth was completely blocked by constitutively active Cdc42, and the outgrowth was suppressed in cells expressing constitutively active Rac1 or either dominant negative variant (Fig. 1C). As for full-length PKCε, the attenuating effect was larger for dominant negative Cdc42 than for the corresponding Rac1 mutant.

To further investigate the role of Rho GTPases in neurite outgrowth dependent on PKC, we studied their effect on the neurite outgrowth during neuronal differentiation. Two differentiation protocols, treatment of SK-N-BE(2) cells with RA or of SH-SY5Y/TrkA cells with NGF, were utilized. For both protocols the involvement of the regulatory domain of PKCε has been shown to be important for the morphological effects [9,10]. In RA-treated SK-N-BE(2) cells, which had been transfected with EGFP-vector alone, ~40% of the cells had neurites. Expression of either constitutively active Rac1 or Cdc42 completely blocked the formation of neurites, while both dominant negative mutants suppressed the outgrowth (Fig. 1D). A similar effect of Rac1 and Cdc42 was seen in NGF-treated SH-SY5Y/TrkA cells, with the constitutively active mutants blocking the neurite formation and the dominant negative mutants having a suppressive effect (Fig. 1E). For both differentiation protocols, the inhibiting effects of dominant negative Rac1 and Cdc42 were similar in magnitude. As for the PKCε experiments, only cells positive for both EGFP and the myc-tag were included, ensuring that all cells analysed expressed the Rac1 and Cdc42 mutants.

The effect of Rac1 and Cdc42 on neurite outgrowth induced by the PKCε regulatory domain was alsoexamined

in immortalized hippocampal (HiB5) cells, (Fig. 1F). In line with the results seen in neuroblastoma cells, both constitutively active mutants blocked the formation of neurites, while the dominant negative mutants had a suppressive effect. Furthermore, the magnitude of the suppression was larger for dominant negative Cdc42 than for dominant negative Rac1. Neither in these cells did either mutant of Rac1 and Cdc42 induce the formation of neurites on its own.

For neuroblastoma cells, only cells staining positive for the myc-tag were analysed, ensuring that expression levels above a certain threshold were obtained for all mutants in the examined cells. In HiB5 cells, only EGFP expression was used as a marker for transfected cells. We therefore analysed the expression levels of the myc-tagged GTPases in HiB5 cells with Western blot (Fig. 1G). This demonstrated that constitutively active Cdc42 was expressed at markedly higher levels than the active Rac1 variant but both constructs completely ablated the neurite outgrowth making it impossible to draw any conclusions regarding differential effectiveness of them. More importantly, the dominant negative variants were expressed at similar levels indicating that the more prominent effect of dominant negative Cdc42 is not due to differences in expression levels. Taken together, dominant negative Cdc42 has a more attenuating effect on PKCε-induced neurite outgrowth than the corresponding Rac1 mutant. However, for RA- and NGF-stimulated outgrowth their inhibitory effect is similar.

Constitutively active and dominant negative Cdc42 inhibit PKC-mediated stress fibre loss in HiB5 cells

We have seen that HiB5 cells overexpressing PKCδ can be induced to extend neurites upon treatment with phorbol esters [11]. As for PKCε, this effect is independent of the catalytic activity and mediated via the regulatory domain of the protein. Using PKCδ-overexpressing cells as model system, we have seen that the PKC-mediated outgrowth of neurites is accompanied by a loss of stress fibres. To elucidate if Rac1 and Cdc42 are involved in this morphological PKC effect as well, we studied the TPA-induced dismantling of stress fibres in HiB5 cells overexpressing PKCδ together with the Rac1 or Cdc42 mutants. HiB5 cells were co-transfected with an expression vector encoding EGFP-tagged PKCδ together with vectors encoding myc-tagged constitutively active or dominant negative Rac1 or Cdc42, or with an empty myc-vector as control (Fig. 2). In cells transfected with PKCδ-vector together with empty myc-vector, complete stress fibre loss was induced after 15 min of TPA treatment (Fig. 2A and B). Expression of constitutively active Rac1 induced a profound morphological effect by itself with the formation of lamellipodia and a reduction in the amount of stress fibres in the cell (Fig. 2A). The PKCδ-induced stress fibre loss was partially inhibited in cells expressing the constitutively active Rac1, while the dominant negative variant had no effect. In contrast,

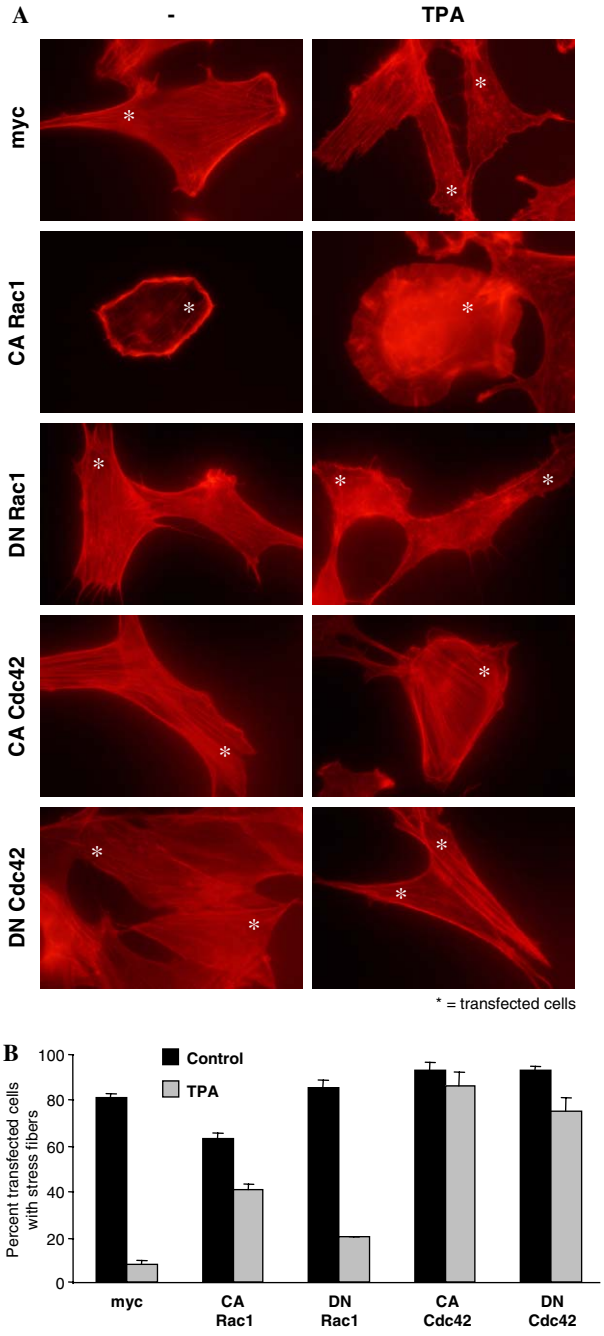

Fig. 2. Cdc42 mutants inhibit PKCδ-mediated stress fibre loss in HiB5 cells. HiB5 cells were co-transfected with a vector encoding PKCδ fused to EGFP together with either an empty myc-vector or a vector encoding myc-tagged constitutively active or dominant negative Rac1 or Cdc42. Sixteen hours after transfection, cells were treated for 15 min with 16 nM TPA, fixed, and stained with Alexa Flour 546-conjugated phalloidin to visualize F-actin. (A) Fluorescent images showing F-actin cytoskeleton in TPA-treated HiB5 cells overexpressing PKCδ together with indicated GTPase mutant. * = transfected cells. (B) Quantification of the number of TPA-treated PKCδ-overexpressing cells with stress fibres. Data (means ± SEM, $n = 3$) are percentage of transfected cells with stress fibres.

expression of both the dominant negative and constitutively active Cdc42 prevented the TPA-induced stress fibre loss (Fig. 2A and B). Thus, as for PKC-mediated neurite outgrowth, PKC-induced stress fibre disassembly is more sen-

sitive to dominant negative Cdc42 than to the corresponding Rac1 variant.

Cdc42 mutants inhibit neurite outgrowth, but not stress fibre loss, induced by Y-27632 treatment

The formation of stress fibres is regulated by RhoA and its effector ROCK [20–22]. Inhibition of ROCK leads to both stress fibre dismantling and neurite outgrowth in HiB5 cells [11]. Furthermore, RhoA inhibits both PKC-mediated neurite outgrowth and stress fibre dismantling [11], suggesting that an attenuation of the RhoA pathway may be important for the PKC effects. The previous results indicate that Cdc42 is important for both PKC-induced neurite outgrowth and stress fibre loss. Cdc42 may be a link between PKC and suppression of the RhoA pathway. If this is the case, morphological effects induced by inhibition of the RhoA pathway should be insensitive to dominant negative Cdc42. We therefore studied the effects of the Cdc42 mutants on neurite outgrowth and stress fibre dismantling induced by a direct inhibition of the RhoA effector ROCK. HiB5 cells were co-transfected with either empty myc-vector or vectors encoding constitutively active or dominant negative Cdc42, together with an empty EGFP-vector to be able to detect transfected cells. Sixteen hours after transfection, cells were treated for 15 min with the ROCK inhibitor Y-27632. As seen in Fig. 3A, neither constitutively active nor dominant negative Cdc42 inhibited Y-27632-induced stress fibre dismantling to the same degree as for the PKC-mediated disassembly (Fig. 2B). On the other hand, both mutants substantially suppressed the outgrowth of neurites induced by treatment with Y-27632 for 16 h (Fig. 3B).

Rac1 and Cdc42 are important for PKC-induced outgrowth of beaded extensions in fibroblasts

We have previously shown that overexpression of PKCε, and to a lesser extent PKCδ, induces the formation of beaded extensions in NIH 3T3 fibroblasts [12]. Considering that the dynamics of the actin cytoskeleton have been widely studied in fibroblasts, we investigated whether the Cdc42 and Rac1 mutants influence the PKC-induced morphological alterations also in these cells. Fibroblasts were therefore co-transfected with vectors encoding the regulatory domain of PKCε together with either empty myc-vector or vectors encoding constitutively active or dominant negative Rac1 or Cdc42. As seen in Fig. 4A, both constitutively active mutants blocked the outgrowth, while dominant negative Cdc42 had a suppressive effect. Dominant negative Rac1 did not affect the outgrowth of beaded extension. The expression levels of the Rac1 and Cdc42 mutants in fibroblasts were investigated with Western blot (Fig. 4B). The results show that the dominant negative mutants were expressed at similar levels and in larger amounts than the constitutively active mutants.

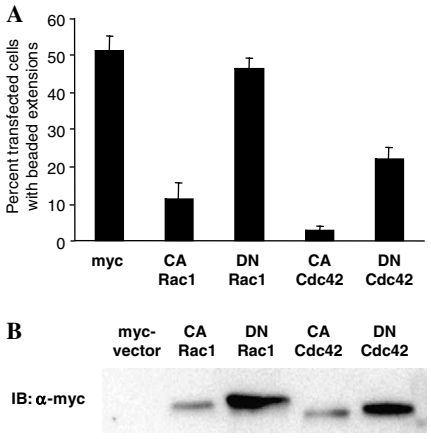

Fig. 4. Cdc42 mutants and constitutively active Rac1 suppress PKCε-mediated outgrowth of beaded extensions in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were co-transfected with a vector encoding the regulatory domain of PKCε (PKCεRD) fused to EGFP together with either an empty myc-vector or a vector encoding myc-tagged constitutively active or dominant negative Rac1 or Cdc42. (A) Sixteen hours after transfection, cells were fixed, mounted, and transfected cells were scored for the presence of beaded extensions. Data (means ± SEM, $n = 3$) are percentage of transfected cells with neurites. (B) Western blot on lysates from NIH 3T3 fibroblasts transfected with either myc-vector or a vector encoding myc-tagged constitutively active or dominant negative Rac1 or Cdc42 using an antibody toward the myc-tag demonstrates expression levels of the different mutants.

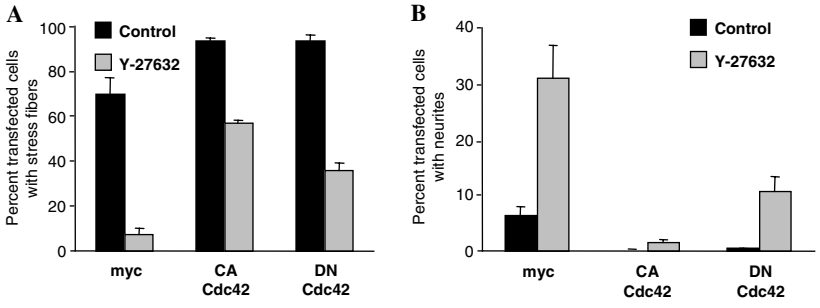

Fig. 3. The effect of Cdc42 mutants on stress fibre loss and neurite outgrowth induced by ROCK inhibition. HiB5 cells were co-transfected with EGFP-vector and either myc-vector or a vector encoding myc-tagged constitutively active or dominant negative Cdc42. Sixteen hours after transfection, cells were treated with 10 μM Y-27632 for 15 min, fixed, and stained with Alexa Flour 546-conjugated phalloidin to visualize F-actin. EGFP-positive cells with stress fibres (A) or neurites (B) were counted. Data (means ± SEM, $n = 3$) are percentage of transfected cells with stress fibres or neurites.

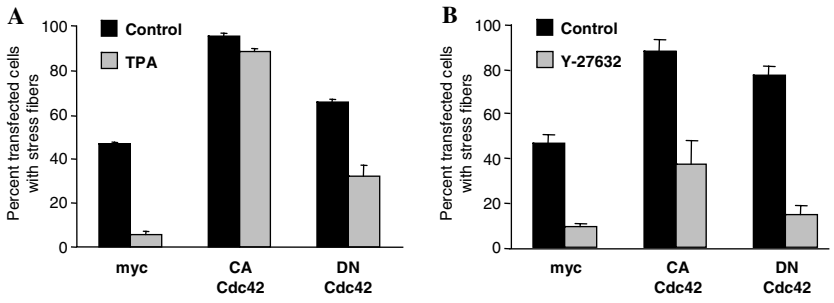

Fig. 5. Cdc42 mutants attenuate PKCδ-mediated stress fibre loss in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were co-transfected with either myc-vector or vector encoding myc-tagged constitutively active or dominant negative Cdc42 together with (A) a vector encoding PKCδ fused to EGFP or (B) EGFP-vector only. Sixteen hours after transfection, cells were treated for 15 min with (A) 16 nM TPA or (B) 10 μM Y-27632, fixed, and stained with Alexa Flour 546-conjugated phalloidin to visualize F-actin, and scored for the presence of stress fibres. Data (means ± SEM, $n = 3$) are percentage of transfected cells with stress fibres.

Constitutively active Cdc42 blocks stress fibre loss in fibroblasts

We next investigated if the Cdc42 mutants had the same effect on TPA-induced stress fibre loss in fibroblasts over-expressing PKCδ as they do in HiB5 cells. Fibroblasts were co-transfected with vector encoding EGFP-tagged PKCδ together with either myc-vector or vectors encoding constitutively active or dominant negative Cdc42. Sixteen hours after transfection, cells were treated with TPA for 15 min. As seen in Fig. 5A, constitutively active Cdc42 inhibited and dominant negative Cdc42 attenuated the loss of stress fibres.

As with the HiB5 cells, we also investigated the effects of Cdc42 on the stress fibre loss induced by inhibiting ROCK in fibroblasts. Cells were co-transfected with either empty myc-vector or vectors encoding constitutively active or dominant negative Cdc42, together with an empty EGFP-vector. Sixteen hours after transfection, cells were treated for 15 min with the ROCK inhibitor Y-27632. The results showed that neither of the Cdc42 mutants blocked the Y-27632-induced disassembly of the stress fibres, in line with the result from the HiB5 cells.

Discussion

Previous studies by our group have shown that PKCε, independently of its catalytic activity, via the regulatory domain induces the formation of long cellular processes in both neural cells [9–11] and in fibroblasts [12]. It is therefore conceivable that the PKC regulatory domains target cytoskeletal regulators that are common for several cell types and important for neurite outgrowth. We have seen that the PKC effects are similar to what happens when the RhoA effector ROCK is inhibited and that RhoA blocks the PKC effect [11]. To further elucidate the mechanisms mediating PKC-induced cytoskeletal changes, we investigated the role of additional members of the Rho family of small GTPases.

Our initial focus was on Rac1 and Cdc42. There are many studies indicating critical roles for these proteins in the induction and elongation of neurites [17,23–28]. Our findings show that both the constitutively active and the dominant negative mutants attenuate the PKC-mediated outgrowth both in neural cells and, with the exception of the dominant negative Rac1, in fibroblasts. This shows that the morphological changes induced by PKC require the activity of both Rac1 and Cdc42, and further strengthens the role for these GTPases in the outgrowth of cellular processes.

The fact that the active mutants of these proteins essentially abolished the PKC-induced outgrowth of processes and neurites in all cells studied could at first sight be counterintuitive. However, this effect of the active mutants has also been reported in other model systems [26,28,29]. It is conceivable that a dynamic cellular morphology requires a coordinated cycle of protrusion, attachment, and traction at the leading edge. Each event is regulated by specific GTPases, and thus dependent on the activation of the different GTPases at the right time and place. Locking them in a perpetually active state may therefore alter the balance of active GTPase and by strongly promoting a specific cytoskeletal feature disrupt the necessary dynamics of the actin cytoskeleton and thereby stalling the change in morphology.

Both constitutively active and dominant negative Cdc42 had generally larger suppressive effect than corresponding Rac1 mutants. Considering that the expression levels of the dominant negative constructs were similar in both HiB5 cells and fibroblasts, and that the levels of constitutively active mutants were similar in fibroblasts, this effect does not seem to be due to different expression levels. Thus, Cdc42 seems to have a more prominent role than Rac1 in the pathway that mediates PKC-induced formation of long cellular processes.

As for PKC-induced neurite outgrowth, constitutively active Cdc42 inhibited the TPA-induced stress fibre loss in both HiB5 cells and fibroblasts. In HiB5 cells, the constitutively active Rac1 on its own had a profound effect on cell morphology which makes it difficult to asses its role in PKC-mediated effects. The dominant negative Rac1 had no effect whereas dominant negative Cdc42 abolished

the PKC-mediated stress fibre dismantling in HiB5 cells and suppressed it in fibroblasts. Thus, as for neurite outgrowth, Cdc42 seems to be the more important of the two GTPases in terms of mediation of the PKC effect.

There are several possible mechanisms through which Cdc42 could act to dismantle stress fibres. For example, Cdc42 has been shown to depolymerize actin filaments via the cofilin homology domain of N-WASP [30,31]. Cdc42 has also been shown to inhibit the activity of RhoA [32–34], which is the GTPase mainly responsible for the formation of stress fibres via the effector ROCK [21,22,35]. Furthermore, Cdc42 may also lead to the disassembly of stress fibres via the effector protein Pak1, which has been shown to counteract the phosphorylation of the myosin light chain [36], which is necessary for the formation of stress fibres and induced by the RhoA/ROCK pathway [35,37].

Contrasting their effects on PKC-mediated stress fibre disassembly, the Cdc42 mutants had smaller effects on the loss of stress fibres induced by treating cells with the ROCK inhibitor. This demonstrates that Cdc42 is not necessary for stress fibre disassembly per se. Instead Cdc42 may be more involved in the pathway by which PKC induces the dismantling.

In line with their effects on PKC-induced outgrowth of cellular processes, the Cdc42 mutants markedly suppressed the same effect of Y-27632. In fact, the Cdc42 mutants influenced neurite outgrowth induced by all agents that were investigated. Thus, Cdc42 may influence the PKC-induced morphological changes at two levels, both in a specific pathway mediating PKC-induced stress fibre disassembly and as a general prerequisite for the outgrowth of cellular processes. This may explain why the PKC-induced outgrowth is particularly sensitive to interference with Cdc42 activity.

The question whether Cdc42 influences the stress fibres directly via an effector or if it suppresses the activity of RhoA remains to be clarified. Measuring the activity of both Cdc42 and RhoA in TPA-treated HiB5 cells and fibroblasts overexpressing PKCδ has given inconclusive results due to low transfection efficiency.

In conclusion, our data support crucial roles for both Rac1 and Cdc42 in the induction of cellular processes by PKCε and for Cdc42 in the PKC-induced loss of stress fibres. The effects seem to be general since they were observed both in fibroblasts and in neural cells. Cdc42 may have two roles in mediating the PKC effects. One, which seems to be specific for the PKC pathway, is to mediate the loss of stress fibres and thus a putative counteraction of the RhoA pathway, and one as a crucial component in the induction of neurite outgrowth irrespective of the stimulus.

Acknowledgments

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