Dual lipidation of the brain-specific Cdc42 isoform regulates its functional properties

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INTRODUCTION

The family of Ras homology (Rho) small GTPases consists of more than 20 signalling proteins in humans, with Cdc42 (cell division cycle 42), RhoA and Rac1 being the best-studied members of the Rho family. Although Rho GTPases are involved in the regulation of a wide range of cellular activities, their more prominent function is the regulation of actin and microtubule cytoskeleton in a variety of cell types [1–4]. Rho GTPases are also expressed in multiple neural tissues, where they act as key mediators that link the extracellular signals to cytoskeletal rearrangements. Multiple studies in neurons suggest that Cdc42 is a positive regulator promoting neurite outgrowth, dendritic branching, axonal pathfinding and neuronal motility. Moreover, Cdc42 appears to be a part of the initial molecular cascade required for the growth of new synapses [5–8]. Cdc42 is also involved in transcriptional regulation via activation of specific transcription factors, such as SRF (serum-response factor), which binds to the SRE (serum-response element) [9,10]. This results in an activation of transcriptional activity of some immediate early genes such as c-fos, nurr and c-Jun. The broad distribution of Cdc42 is paralleled by the existence of two Cdc42 splice variants, which share the same amino acid sequence up to Gln181, whereas the last ten C-terminal located amino acids are isofrom-specific (Figure 1A, [11]).

Physiological functions of small GTPases can only be achieved when these proteins are correctly distributed within the cell, and their plasma membrane localization is particularly important for their proper functional accomplishment. So far, at least two intrinsic signals are known to be involved in plasma membrane targeting [12]. The first signal includes a stretch of polybasic amino acids located within the hypervariable region, which increase the affinity of proteins to membrane lipids [13,14]. The second important signal targeting members of Ras and Rho GTPases to the plasma membrane is C-terminal prenylation [15]. For instance, the canonical Cdc42 isoform contains at least three basic amino acids localized close to its C-terminus (Figure 1A), and this isoform has been shown to be prenylated (Cdc42-prenyl) [15]. In addition, GTPases are known to undergo post-translational palmitoylation within their hypervariable region [16,17]. Palmitoylation is a reversible attachment of the C16 saturated fatty acid palmitate to cysteine residue(s) via thioester linkage. It has been shown that the non-canonical brain-specific isoform of Cdc42 can be palmitoylated (Cdc42-palm) [18]. Palmitoylation occurs both in the Golgi compartment as well as directly at the plasma membrane [19] and is mediated by specific PATs (palmitoyl acyl-transferases) belonging to the DHH-containing zinc finger protein family [20]. It has been suggested that repeating palmitoylation–depalmitoylation cycles can represent an important molecular mechanism responsible for the dynamic translocation of cytosolic signalling proteins, including small GTPases, to the plasma membrane [21–23].

In the present study we have demonstrated that the non-canonical brain-specific Cdc42-palm isoform is palmitoylated
on Cys\textsuperscript{188} and Cys\textsuperscript{189} both in vitro and in vivo. We have also found that, in addition to palmitoylation, Cys\textsuperscript{188} can be isoprenylated by both GGTase\textsubscript{I} (geranylgeranyltransferase \textsubscript{I}) and FTase (farnesyltransferase). Lipidation of Cys\textsuperscript{188} is essential for the plasma membrane localization of Cdc42-palm and is critically involved in Cdc42-mediated regulation of gene transcription and cellular morphology. Expression analysis revealed that Cdc42-prenyl is a dominant isoform in a wide range of commonly used laboratory cell lines as well as in the cerebellum, whereas Cdc42-palm is highly expressed in the hippocampus. Functionally, lipidation of Cdc42-palm plays an important role in induction of dendritic protrusion and formation of dendritic spines.

**EXPERIMENTAL**

**Antibodies**

A polyclonal rabbit anti-GFP antibody was purchased from GeneTex and a HRP (horseradish peroxidase)-conjugated antibiotin antibody was purchased from Sigma–Aldrich. Anti-Cdc42 monoclonal antibody was purchased from BD Biosciences.

**Cell culture and transfection**

The murine neuroblastoma cell line N1E-115 was cultured and maintained in DMEM (Dulbecco’s modified Eagle’s medium)
Table 1  Primer list for site-directed mutagenesis of Cdc42

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palm/Prenyl G12V_for</td>
<td>GATGTCATTGCTCGGAGATTTTACAGCACAGCACAGCAC</td>
</tr>
<tr>
<td>Palm/Prenyl G12V_rev</td>
<td>GACTATTTTACACACAGCACAGCACAGCACAGCACAGCAC</td>
</tr>
<tr>
<td>Palm/Prenyl T17N_for</td>
<td>GATGTCATTGCTCGGAGATTTTACAGCACAGCACAGCAC</td>
</tr>
<tr>
<td>Palm/Prenyl T17N_rev</td>
<td>GACTATTTTACACACAGCACAGCACAGCACAGCACAGCAC</td>
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<tr>
<td>Palm C188A_for</td>
<td>CGAGCTCCGGAAAATCTACACACAAAAGGAGGTCGATATCT</td>
</tr>
<tr>
<td>Palm C188A_rev</td>
<td>AGAATACAGCGCTTTTGGGTTGGTCCGGAGGCCGTCG</td>
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<tr>
<td>Palm C189A_for</td>
<td>CGAGCTCCGGAAAATCTACACACAAAAGGAGGTCGATATCT</td>
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<tr>
<td>Palm C189A_ref</td>
<td>AGAATACAGCGCTTTTGGGTTGGTCCGGAGGCCGTCG</td>
</tr>
<tr>
<td>Palm C188/189A_for</td>
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<td>Palm C188/189A_rev</td>
<td>AGAATACAGCGCTTTTGGGTTGGTCCGGAGGCCGTCG</td>
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<tr>
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<td>GAGGCTCCGAGACACAGAGAGGCGGCTGTCGCTAT</td>
</tr>
<tr>
<td>Prenyl C188A_rev</td>
<td>ATACGACGACAGCGCTTGGCCGCTTTGCCTGCGGCTCG</td>
</tr>
<tr>
<td>Prenyl palm-C_for</td>
<td>GAGGCTCCGAGACACAGAGAGGCGGCTGTCGCTAT</td>
</tr>
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<td>Prenyl palm-C_rev</td>
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<tr>
<td>Prenyl palm-C188A_rev</td>
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<tr>
<td>Prenyl palm C189A_for</td>
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<tr>
<td>Prenyl palm C189A_rev</td>
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<tr>
<td>Prenyl/Palm T17N_for</td>
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<td>Prenyl/Palm G12V_rev</td>
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<td>Palm/Prenyl C189A_rev</td>
<td>ATACGACGACAGCGCTTGGCCGCTTTGCCTGCGGCTCG</td>
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Plasmids and mutagenesis

Two isoforms of murine Cdc42 (Cdc42-prenyl and Cdc42-palm) were cloned from mouse brain cDNA into pcDNA3.1(+) vector after fusion with GFP to the N-terminus. All Cdc42 mutants were derived from either pcDNA3.1(+) containing Cdc42-prenyl-GFP or pcDNA3.1(+) containing Cdc42-prenyl-GFP. Site-directed mutagenesis was performed using the QuikChange® site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene). Primers used for mutagenesis are summarized in Table 1. The CA (constitutively active) Cdc42 mutant used was the GTP-hydrolysis deficient G12V mutant.

Adeno-associated virus production

The original expression plasmid AAV-Syn(0.5)-EGFP was a gift from Dr Pavel Osten (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.). We modified the vector by introducing the H1 promoter into the Mslu site for the expression of shRNA, and shRNA-resistant Cdc42-palm and Cdc42-prenyl variants were introduced in the same vector (Supplementary Figure S1 at http://www.biochemj.org/bj/456/bj4560311add.htm). Thus our vectors contain two independent expression cassettes: H1 for the production of shRNA and Cdc42–EGFP mutants driven by the human Synapsin-0.5(Syn) promoter [24]. To inhibit both isoforms of Cdc42, we used a shRNA sequence designed at 5′ from ORF. Constructs with Cdc42-specific shRNAs were transfected in NIE cells and tested by real-time PCR. The most effective shRNA (sh4) was chosen (5′-CTGTTTCCGAAATGTCGAC-3′). AAV (adeno-associated virus) production was performed in HEK (human embryonic kidney)-293FT cells using cotransfection of the main expression plasmids with pDP1 and pDP2 helper plasmids [25]. After purification, the recombinant viruses were titrated by real-time PCR using a WPRE (woodchuck hepatitis virus post-transcriptional regulatory element)-specific sequence. For control experiments the construct expressing scramble shRNA (5′-ACTACCGTTTATAGGTG-3′) and EGFP was used.

In vitro prenylation assay

MBP (myelin basic protein)-tagged Cdc42-palm, Cdc42-palm C188A, Cdc42-palm C189A and Cdc42-prenyl were expressed in Escherichia coli and purified by Ni-NTA (Ni2+-nitrilotriacetaate) affinity chromatography and size-exclusion chromatography. Prenylation reactions were performed with fluorescent analogues of lipid substrates, i.e. NBD-GFP as FTase substrate (FPP), NBD-FPP as GGTaseI substrate (GGPP). A mixture of 6 μM Cdc42 protein, 6 μM FTase or GGTaseI and 40 μM NBD-GFP or NBD-FPP in the prenylation buffer (50 mM Hepes, pH 7.2, 50 mM NaCl, 2 mM MgCl2 and 2 mM dithioerythritol) were incubated for 1 h at 25 °C. The reaction mixtures were resolved by SDS/PAGE (15% gels) and the gel was scanned using a Fluorescent Image Reader FLA-5000 (Fujifilm) (excitation laser at 473 nm and emission cut-off filter at 510 nm). The fluorescent bands corresponding to the formation of the prenylated product were quantified using the AIDA densitometry software.

ABE (acyl-biotinyl exchange) assay

NIE-115 cells transfected with GFP-tagged Cdc42 were lysed in buffer containing 10 mM N-ethylmaleimide and proteins were precipitated with methanol/chloroform extraction. Cell lysates were divided into two portions. One portion was treated with 0.5 M hydroxyamine (pH 7.35) and the other portion with 30 mM Tris/HisCl (pH 7.4) which was used as control. At the same time proteins were labelled with HPDP-biotin {N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide]. Afterwards cell lysates were immunoprecipitated with polyclonal rabbit anti-GFP antibody and Protein A–Sepharose. After washing, the protein complex was eluted and lysates were subjected to SDS/PAGE and Western blotting. Total Cdc42 was detected with HRP-tagged anti-GFP antibody, whereas palmitoylated Cdc42 was detected with HRP-tagged anti-biotin antibody. Images were taken by PeqLab FUSION SL and quantified with ImageJ (NIH). To inhibit the

containing 10% FBS and 100 units of penicillin/streptomycin at 37°C and 5% CO2. To analyse recombinant proteins, NIE-115 cells were transfected with the corresponding plasmids using Lipofectamine® 2000 reagent (Life Technologies) according to the manufacturer’s instructions.

Hippocampal neuron cultures were prepared from National Maritime Research Institute (NMRI) mice at postnatal day 1 according to an optimized protocol for mouse hippocampal neurons [7]. All animals were housed, cared for and killed in accordance with the recommendations of the European Commission.

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prenylation, FTase inhibitor I and GGTL-2147 were used at a concentration of 50 μM and incubated for 16 h.

A modified ABE protocol was used to analyse palmitoylation of Cdc42 in native brain tissue from mice, rats and humans as described previously [26]. The study of Cdc42 palmitoylation in human brains was performed in the samples from prefrontal cortex (Brodmann area 9) of non-psychiatric control subjects. Brain tissues were obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center, Baltimore, MD, U.S.A. Brain tissue was collected only after a family member gave informed consent. All procedures were approved by the Institutional Review Boards of the University of Maryland and the University of Illinois at Chicago.

FRAP
FRAP experiments were carried out with Zeiss LSM780 confocal microscope. The pinhole was set to two airy units to reduce the focal volume and to collect the fluorescence from a narrow depth in the focal plane, enabling us to measure fluorescence intensity from the plasma membrane. The fluorescence was excited with an Argon laser at 488 nm wavelength. To bleach a defined ROI within the cell, a 405 nm diode laser (50 mW) was used at 100% of laser intensity. After baseline acquisition, the recovery phase was traced and bi-exponentially fitted offline. Recovery halftime was calculated after normalization, background and reference correction according to Kenworthy et al. [27].

Confocal imaging and morphometric analysis
N1E-115 cells were plated on 18-mm-diameter coverslips and transiently transfected with corresponding Cdc42-encoding plasmids. After overnight incubation, cells were subjected to confocal imaging and z-stack acquisition using Zeiss LSM780. Morphometric analysis of transfected N1E-115 cells was performed as a double-blind test and evaluated using Fiji ImageJ and MoticPlus 2.0 softwares.

For morphometric analysis of hippocampal neurons, neuronal cultures were infected with AAVs at DIV8 (day in vitro 8) at the multiplicity of infection of 1000 and cultured for 7 days post-infection. Imaging of hippocampal neurons was carried out with a Zeiss LSM780 at DIV5. Numbers of LPs (long dendritic protrusions) (>10 μm) and SPs (short dendritic protrusions) (<10 μm) per 50 μm of hippocampal neurons were analysed with MoticPlus 2.0 and Imaris Software as a double-blind experiment [7].

Dual luciferase reporter assay
To measure the activation of gene expression controlled by SRF we used a dual-luciferase® reporter assay in a 96-well format, and the luminescence was quantified with a Mithras LB 940 system. The PGL4.34[lu2P/SRF-RE/Hygro] plasmid was used as the reporter and pGL4.50[lu2C/CMV/Hygro] was used as control. Both plasmids were co-transfected in N1E-115 cells together with indicated Cdc42 isoforms or mutants. Cells were analysed 16 h after transfection as described previously [28,29].

Real-time PCR
To analyse expression level of different Cdc42 isoforms, total RNA was isolated from different mouse brain regions with TRIzol® followed by cDNA synthesis using SuperScript® III First-Strand Synthesis System according to the manufacturer’s instructions. Primers [FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethylrhodamine) labels] were ordered as TaqMan Gene Expression probes. The custom-made primer probe sets were the following: common, 5’-AGAAAAATGG-GTTGCGTGGAT-3’ (forward), 5’-AAATTGGTCCACCCACC-AACGCAA-3’ (reverse), and 6-FAM-CTCACCCTGTCCCA-AACTCTTCT-TAMRA (probe); and Cdc42-prenyl, 5’-GGCT-CCACACCCAAAGAAGA-3’ (forward), 5’-ACACACGTGTCG-CAGAAAGG-3’ (reverse), and 6-FAM-CGCCAGGTTGTGG-CTGCTATG-TAMRA (probe); Cdc42-palm, 5’-AAAGATGT-GTTGTGATAGGCTATCCT-3’ (forward), 5’-GTGGTAGATA-TACGACACTCTTTGGG-3’ (reverse), and 6-FAM-CCC-TCGAGCTTCGAAACTC-TAMRA (probe). Amplification reaction and analysis have been described previously [30].

Statistical analysis
For statistical analysis, GraphPad Prism 5.0 software was used. Results are expressed as means ± S.E.M. For ABE, FRAP and expression analysis, a Bonferroni post-test corrected unpaired two-tailed t test was applied. Multiple comparisons were carried out using two-way ANOVA. The significance level was chosen as P = 0.05.

RESULTS
Brain-specific Cdc42 isoform is palmitoylated at Cys188 and Cys189
It is known that Cdc42 can be expressed as both a canonical protein as well as non-canonical brain-specific splice variant (Figure 1A). The first isoform is prenylated (Cdc42-prenyl) at its C-terminal CaaX motif (in which C is cysteine, a is preferably an aliphatic amino acid and X is any amino acid), whereas the second isoform can be post-translationally palmitoylated (Cdc42-palm) in cultured neurons as well as after overexpression in HEK-293 cells [18,31]. Using a modified protocol of an ABE approach, we found that endogenous Cdc42 undergoes palmitoylation in mouse, rat and human brains (Figure 1B).

Cdc42-palm exhibits two C-terminal cysteine residues (Figure 1A) representing potential acylation sites. We thus individually or jointly mutated these residues, Cys188 and Cys189 to alanine and tested the consequences on Cdc42 acylation. Palmitoylation level of Cdc42-prenyl isoform does not incorporate palmitic acid, although it contains a cysteine residue at position 188 (results not shown, Figure 1A). We analysed molecular features responsible for specific palmitoylation of the Cdc42-palm isoform. For that, we created two chimaeric proteins in which the last three C-terminal amino acids of Cdc42-palm were replaced with those derived from Cdc42-prenyl (Cdc42-palm/prenyl-C chimaera) and vice versa (Cdc42-prenyl/palm-C chimaera; Figure 1A). As shown in Figures 1(E) and 1(F), the Cdc42-palm/prenyl-C construct was not palmitoylated. In contrast, the palmitoylation level of Cdc42-prenyl/palm-C chimaera was even higher than that obtained for the Cdc42-palm isoform, suggesting that the last three C-terminal amino acids are critically involved in Cdc42 acylation.

Cdc42-palm isoform is prenylated in addition to palmitoylation
To study whether Cdc42-palm can be prenylated, we applied an in vitro prenylation assay after overexpression of MBP-tagged Cdc42-palm and Cdc42-prenyl proteins along with
suggesting that the post-translational lipidation of Cys188 plays a major role in regulation of Cdc42-palm mobility. In contrast, the recovery time for Cdc42-prenyl, \( \tau \), and for Cdc42-palm, \( \tau \), is solely palmitoylated.

**Distribution dynamics of Cdc42-palm is regulated by lipidation of Cys\(^{188} \)**

To study the role of palmitoylation and prenylation of Cdc42 in regulation of its intracellular distribution, we used FRAP experiments after transfection of mouse neuroblastoma N1E-115 cells with GFP-tagged Cdc42 wild-type or its cysteine mutants (Figures 3A and 3B). As Cdc42 is a peripheral membrane protein, its fluorescence recovery in FRAP experiments represents a combination of both lateral diffusion and dynamic exchange between the membrane-bound and cytosolic pool. After photobleaching, Cdc42-palm fluorescence recovered significantly faster than that of Cdc42-prenyl (Figure 3C; half-time for recovery of Cdc42-palm, \( \tau_{1/2} = 5.36 \pm 0.29 \) s, \( n = 24 \) and for Cdc42-prenyl, \( \tau_{1/2} = 7.22 \pm 0.65 \) s, \( n = 14 \)), suggesting that the Cdc42-palm is highly dynamic. To test whether Cdc42 recovery can be regulated by its post-translational lipidation, we performed FRAP experiments on cysteine residue mutants of the Cdc42-palm isoform. The recovery time for C188A (\( \tau_{1/2} = 3.64 \pm 0.33 \) s, \( n = 12 \)) and C188A/C189A (\( \tau_{1/2} = 4.02 \pm 0.30 \) s, \( n = 20 \)) mutants was significantly faster than that of Cdc42-palm (Figure 3C). The results are the means \( \pm \) S.E.M. from at least three independent experiments (**P < 0.01; ***P < 0.001). WT, wild-type.

**Lipidation of Cys\(^{188} \) of Cdc42-palm isoform is necessary for formation of densely packed filopodia in neuroblastoma cells**

To study the functional role of Cdc42 lipidation in the regulation of cellular morphology, we overexpressed GFP-tagged Cdc42 constructs in neuroblastoma cells followed by morphometric analysis. As illustrated in Figure 4(A), Cdc42-palm and Cdc42-prenyl are preferentially localized within the Golgi compartment and at the plasma membrane. In addition, Cdc42-palm shows a stronger appearance in the near front of protrusions. Overexpression of either Cdc42-palm or Cdc42-prenyl strongly promotes the formation of the densely packed filopodia, and there was no significant difference between both Cdc42 isoforms observed (Figure 4B). When the CA Cdc42 mutant G12V (Cdc42 CA) was overexpressed, a further increase in the number of densely packed filopodia was obtained (Cdc42-palm CA, 27.59 \( \pm \) 3.11 compared with Cdc42-prenyl CA, 22.79 \( \pm \) 2.10). These data demonstrate that, as long as Cdc42 is properly lipidated, their activation state is mainly responsible for the Cdc42-mediated regulation of actin cytoskeleton organization and assembling filopodia. Mutation of Cys\(^{188} \), either alone or in combination with Cys\(^{189} \), results in drastic changes of intracellular Cdc42-palm distribution.

![Figure 2](image-url)
membrane localization of both mutants was completely lost and replaced by diffuse cytosolic and nuclear distribution, similar to that obtained for the soluble GFP (Figures 4A and 4C). The ability of Cdc42-palm to induce the formation of densely packed filopodia was also completely abolished and cannot be recovered even by introduction of the CA mutation (Figures 4C and 4D). In contrast, replacement of Cys188 influences neither intracellular distribution nor induction of densely packed filopodia (Figures 4C and 4D). Moreover, combining C189A with the CA mutation leads to a significant increase in the amount of densely packed filopodia (Figures 4C and 4D). These data suggest that lipidation of Cys188 is necessary for the proper intracellular localization and represents an essential prerequisite for the functional activation of the Cdc42-palm isoform by GTP loading.

Regulation of SRE-mediated gene expression by Cdc42-palm is lipidation-dependent

In addition to its effect on the actin cytoskeleton, Cdc42 is known to be involved in the regulation of gene transcription control by activation of the SRE via the SRF [9]. To monitor the role of Cdc42 lipidation in SRE-mediated transcription, neuroblastoma N1E cells were transiently transfected with different Cdc42 constructs along with an SRE-driven luciferase reporter construct, and luciferase activity was measured 16 h post-transfection. As shown in Figure 5, expression of Cdc42-palm and Cdc42-prenyl resulted in an increased SRE activation. It is remarkable that the ability of Cdc42-palm to activate SRE was significantly enhanced in comparison with Cdc42-prenyl, suggesting a higher efficiency of the Cdc42-palm isoform towards the transcriptional regulation. CA mutants of both Cdc42 isoforms caused a drastic increase in SRE activation (Figure 5), whereas the dominant-negative mutants were not able to increase the SRE activity above the basal level (results not shown). Mutation of Cys188, either alone or in combination with Cys189, led to complete abolishment of SRE activation mediated by Cdc42-palm, and this effect was independent of the presence or absence of CA mutation (Figure 5). Notably, the level of SRE activation induced by the C189A mutant was significantly lower compared with the Cdc42-palm wild-type and similar to that obtained for the Cdc42-prenyl wild-type (Figure 5A). Taken together, these results indicate that Cdc42-palm-mediated SRE activation is regulated not only by its GTP-loading status, but critically depends on the lipidation state. In particular, Cys188 (which can be both prenylated and palmitoylated) is necessary to induce SRE activation, whereas palmitoylation of Cys189 seems to be responsible for the fine-tuning of SRE activity. This is further confirmed by the observation that expression of the Cdc42-prenyl/palm-C construct, in which the last three C-terminal amino acids from Cdc42-palm were inserted into Cdc42-prenyl (Figure 1A), was able to induce SRE activation in the same range as Cdc42-palm wild-type (Figure 5).

Role for Cdc42-palm in regulation of neuronal morphology in hippocampal neurons

The experiments presented above demonstrated that recombinant Cdc42-palm and Cdc42-prenyl are involved in the regulation of cellular morphology and gene transcription after overexpression. However, it is still not clear whether the functional preference of a defined isoform can exist under endogenous conditions. To study that, we compared the expression level of both Cdc42 isoforms in different cell types by quantitative PCR. In a broad range of commonly used laboratory cell lines, we obtained a higher expression level of Cdc42-prenyl in comparison with Cdc42-palm ranging from 5-fold (in HEK-293 cells) to 15-fold (in N1E-115 cells, Figure 6A). This suggests a higher functional importance of Cdc42-prenyl isoform in these cells. A similar expression profile with approximately 10-fold higher Cdc42-prenyl expression level was obtained in the cerebellum of mice (Figure 6B). In contrast, the expression level of Cdc42-palm in the hippocampus was approximately 2-fold higher than that of Cdc42-prenyl (Figure 6B), suggesting a functional importance of Cdc42-palm in the hippocampus.

To analyse the functional properties of neuronal Cdc42 in more detail, we generated AAV constructs encoding shRNA to silence both endogenously expressed Cdc42 isoforms. Real-time PCR analysis revealed that infection of primary neuronal cultures from mouse hippocampus with shRNA-encoding AAV vector decreases the expression of Cdc42 to 26 ± 2.2% (Supplementary Figure S1). We also produced bicistronic AAV constructs encoding not only shRNA against Cdc42, but also shRNA-resistant Cdc42-palm or Cdc42-prenyl (Supplementary Figure S1). Using these constructs, we were able to replace the expression of endogenous Cdc42 by either GFP-tagged wild-type or mutants of Cdc42-palm isoforms. Analysis of bicistronic AAV vectors demonstrated their high efficiency to silence expression of the endogenous Cdc42 in neuroblastoma N1E cells, which
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Figure 4  Role of Cdc42 lipidation in regulation of cellular morphology

(A) NIE-115 cells were transiently transfected with both Cdc42 isoforms or with their CA mutants. Representative fluorescence images are shown. An example of the densely packed filopodia is shown as an inset in the palm wild-type (WT) image. Scale bar, 10 μm. (B) Effect of Cdc42 overexpression on formation of densely packed filopodia. The results are the means ± S.E.M. from at least three independent experiments after the blind analysis (*P < 0.05). (C) Role of cysteine mutants of Cdc42-palm for formation of densely packed filopodia was analysed after transfection of corresponding constructs into N1E cells. Representative fluorescence images are shown. Scale bar, 10 μm. (D) Quantification of the number of densely packed filopodia. Cdc42-palm wild-type was set as 100%. C188A, 26.96 ± 6.97%; C188A/C189A, 16.43 ± 2.54%; C188A CA, 19.32 ± 3.00%; C188A/C189A CA, 21.60 ± 5.00%; C189A, 68.47 ± 12.15% and C189A CA, 127.6 ± 12.84%. *P < 0.05; ***P < 0.001.

was accompanied by the overexpression of GFP-tagged shRNA-resistant Cdc42 isoforms (Figure 7A).

To investigate the role for Cdc42 isoforms in dendritic morphogenesis, we used primary cultures of hippocampal neurons. We selected a time window from DIV12 to DIV15, when well-defined synapses are already formed [7,32]. Specific knockdown of endogenous Cdc42 substantially decreased the number of dendritic protrusions compared with neurons expressing scrambled shRNA (control, Figures 7B–7D). The number of SP per 50 μm of dendritic length was 18.84 ± 0.90 in control compared with 14.40 ± 0.71 after Cdc42 knockdown (n = 19, P < 0.001), the number of LPs (over 10 μm) was 1.58 ± 0.14 in control compared with 1.07 ± 0.13 after knockdown of Cdc42 expression by shRNA (n = 19, P < 0.01). When endogenous Cdc42 was replaced by either shRNA-resistant Cdc42-palm or Cdc42-prenyl isoforms, formation of dendritic protrusions was not only rescued, but also significantly increased in comparison with the control (Cdc42-palm: SP, 27.70 ± 1.31; LP, 2.55 ± 0.36. Cdc42-prenyl: SP, 29.28 ± 1.51; LP, 2.32 ± 0.29, n = 16, P < 0.001, Figures 7B–7D). Statistical analysis of dendritic morphology in neurons expressing either C188A or C188A/C189A mutants of shRNA-resistant Cdc42-palm isoform revealed that the number of dendritic protrusions was significantly reduced compared with the neurons expressing scrambled shRNA (C188A: SP, 13.72 ± 1.06; LP, 1.65 ± 0.19; C188A/C189A, SP, 13.19 ± 1.25; LP, 1.70 ± 0.36, n = 10, P < 0.01, Figures 7B–7D). Notably, the C189A mutant was able to increase the number of LPs to the Cdc42-palm level (LP, 2.32 ± 0.24) and to rescue the number of SP to the control level (SP, 18.61 ± 0.66). Taken together, these data demonstrate that
Cdc42-palm represents the dominant Cdc42 isoform in hippocampal neurons which is required for the induction of dendritic filopodia. The post-translational modification of Cys\(^{188}\) is essential for this process, whereas palmitoylation of Cys\(^{189}\) could be involved in the fine regulation of dendritic spine formation.

DISCUSSION

Dual lipidation of Cdc42-palm isoform

In the present study we analysed the lipidation of the small GTPase Cdc42 which is expressed in the form of two splice variants. The canonical Cdc42 isoform is known to be prenylated (Cdc42-prenyl) at Cys\(^{188}\) localized within the C-terminal CaaX motif by a geranylgeranyl moiety [15,33], whereas the non-canonical isoform can be palmitoylated (Cdc42-palm) in cultured neurons as well as after overexpression [18,31]. We demonstrated that the endogenous Cdc42-palm isoform undergoes palmitoylation in rodent and human brains, suggesting the functional role of this modification in vivo. We have also identified Cys\(^{188}\) and Cys\(^{189}\) as potential palmitoylation sites within Cdc42-palm. It is noteworthy that, although Cdc42-prenyl also possesses a cysteine residue at position 188, it cannot be palmitoylated. Since both isoforms differ only in the last ten C-terminal amino acids, this domain seems to be responsible for the selection of lipid moieties attached to Cdc42. Site-directed mutagenesis analysis suggested the particular importance of the last three C-terminal amino acids for palmitoylation, since substitution of the VLL sequence of Cdc42-prenyl for the CIF motif from Cdc42-palm led to very efficient palmitoylation of the resulting chimaera. Accordingly, reciprocal replacement of the CIF motif of Cdc42-palm by the C-terminus of Cdc42-prenyl (VLL) led to complete abolishment of palmitoylation. This suggests that the CIF motif in combination with a stretch of polybasic amino acids in the vicinity of cysteine residues [34] can represent an important recognition signal for palmitoylation of Cdc42-palm isoform. Absence of Cdc42-prenyl palmitoylation can also be explained by blockade of Cys\(^{188}\) with an isoprenoid moiety, which is attached to the protein immediately after protein synthesis in the endoplasmic reticulum [35–37]. Such early prenylation will result in inaccessibility of Cys\(^{188}\) for later palmitoylation in the Golgi compartment and/or at the plasma membrane.

We also addressed the question of putative prenylation of the Cdc42-palm isoform. Therefore we performed an \textit{in vitro} prenylation assay in a bacterial system in order to avoid...
Functional role of dual lipidated Cdc42

Figure 7 Role of Cdc42 lipidation in formation of dendritic filopodia in hippocampal neurons

(A) Endogenously expressed Cdc42 isoforms were efficiently down-regulated with AAV constructs encoding shRNA against Cdc42. Infection of cells with bicistronic AAV constructs encoding shRNA-resistant Cdc42-palm or Cdc42-prenyl along with anti-Cdc42 shRNA results in silencing of the endogenous Cdc42 paralleled by overexpression of GFP-tagged shRNA-resistant Cdc42 isoforms. (B) Representative images showing dendritic morphology of hippocampal neurons infected with different AAV constructs as indicated. Scale bar, 50 μm. (C) The number of SPs (<10 μm) and (D) LPs (>10 μm) was calculated per 50 μm of dendrite. The results are the means ± S.E.M. from at least three independent experiments (*P < 0.05; **P < 0.01). WT, wild-type.

Interference with other lipid modifications existing in eukaryotic cells. One important observation of the present study was that the Cdc42-palm isoform undergoes prenylation at Cys188. In contrast with the Cdc42-prenyl isoform, Cdc42-palm does not seem to discriminate between FTase and GGTaseI, and accepts both C15 and C20 isoprenoids with a similar efficiency. Such differences in the isoprenoid prevalence between Cdc42-prenyl and Cdc42-palm can be explained by differences in the amino acid sequences of their C-terminal CaaX boxes, which are critically involved in the determination of the nature of the prenylation reaction. The C-terminal CaaX box of Cdc42-prenyl (CVLL) contains a leucine residue at the last position, which is consistent with its preferential geranylgeranylation [36–39]. FTase is more flexible and can accept multiple amino acids, including phenylalanine at the last position of the CaaX box [38]. Therefore the C-terminal CCIF of Cdc42-palm can be recognized by both GGTaseI and FTase. This is also in line with results obtained for Ras-related protein TC21 [39] as well as for K-Ras4b and N-Ras [40–42].

Dual post-translational modification by prenylation and palmitoylation has been previously demonstrated in several members of the Ras protein family. For example, N-Ras and H-Ras proteins have been shown to be first farnesylated and then modified with palmitate moieties attached to one or two cysteine residues respectively [16]. Similar post-translational modifications have also been described for Rac1 [43]. However, as far as we know, in all dually lipidated proteins, cysteine residues within the CaaX box are solely prenylated. Moreover, palmitoylated cysteine residues in these proteins are located upstream of the CaaX box, and the aaX motif should be trimmed due to methylation before the protein can be palmitoylated. Thus Cdc42-palm represents the first protein whose Cys188 can be modified by either prenylation or by palmitoylation, whereas the second (palmitoylated) cysteine residue at position 189 is placed within the CaaX box (CCIF) and should therefore bypass methylation excision. During the preparation of the present paper, Nishimura and Linder [31] also reported the dual lipidation of...
Cdc42 by prenylation of Cys\textsuperscript{188} and palmitoylation of Cys\textsuperscript{189}. However, in contrast with our data, the authors didn’t provide experimental evidence for palmitoylation of Cys\textsuperscript{189}. The main reason for that can be the application of different methods for palmitoylation detection. In our study we applied the ABE assay, which allows cumulative detection of palmitoylation on the whole pool of palmitoylated proteins produced after transfection. In the work by Nishimura and Linder [31], the authors used metabolic labelling with 17-ODYA (17-octadeycenoic acid) followed by click reaction and fluorescence detection in gel. Although this method allows analysis of palmitoylation dynamics (which is barely possible by ABE), the sensitivity of this assay is limited by the amount of 17-ODYA incorporated during the labelling time. Moreover, partial loss of 17-ODYA-labelled protein by immunoprecipitation (or chromatography) in combination with the relative moderate efficiency of the cycloaddition (‘click’) reaction can further reduce the sensitivity of this assay and results in a relatively high detection threshold. Accordingly, an 80 % reduction in palmitoylation, which we obtained for both C188A and C189A mutants by ABE (Figure 1C), could be below the detection limit of 17-ODYA labelling approaches. In addition, the relative expression level and/or activity of the DHHC enzyme(s) responsible for Cdc42 palmitoylation can be different in HEK-293 (Nishimura and Linder [31]) and in N1E-115 cells (the present study). This can also result in a different palmitoylation efficiency of Cys\textsuperscript{189}.

Our finding that Cys\textsuperscript{188} can be either prenylated or palmitoylated suggests the existence of two distinct populations of Cdc42-palm isoform: one with solely prenylated (either by farnesylation or by geranylgeranyl) Cys\textsuperscript{188} and palmitoylated Cys\textsuperscript{189} and another one, where both cysteine residues are palmitoylated. The first population can be produced by the initial prenylation of Cys\textsuperscript{188} either by GGTagel or FTase immediately after protein synthesis. This will increase the membrane affinity of resulting protein and thus trigger subsequent palmitoylation of Cys\textsuperscript{188} by specific PATs in Golgi apparatus or at the plasma membrane [21,22]. Since the CCIF motif represents a non-canonical CaaX box, it is reasonable that the portion of Cdc42-palm will not be recognized by prenylating enzymes, allowing subsequent palmitoylation of Cys\textsuperscript{188} (and also Cys\textsuperscript{189}) during protein transport to the plasma membrane. Prenylation and palmitoylation are known to possess different affinity for lipid bilayers [44] and can also target modified proteins to different lipid microdomains [45]. It would therefore be interesting to analyse in future studies whether both populations of Cdc42-palm are differently localized within the cell.

Functional role of the Cdc42-palm lipidation

The distribution of small GTPases is mainly determined by different C-terminal lipid modifications [46]. In the present study we have demonstrated that Cdc42-palm can be both prenylated and palmitoylated. In contrast with prenylation, palmitoylation is the only modification that is reversible, and Cdc42-palm has been shown to undergo dynamic palmitoylation [18]. Thus continuous cycles of re- and de-palmitoylation can be responsible for the dynamic localization of Cdc42-palm to the plasma membrane, whereas Cdc42-prenyl is expected to be stably anchored to the membrane under basal conditions, as it has previously been shown for H-Ras and K-Ras respectively [46]. The dynamic exchange between membrane-bound and cytosolic Cdc42-palm can be further facilitated by its interaction with binding partners such as RhoGDI\textsubscript{a} (Rho guanine-nucleotide-dissociation inhibitor \alpha). Although the results reported by Nishimura and Linder [31] demonstrate that palmitoylation of Cdc42 can negatively regulate its association with RhoGDI\textsubscript{a}, the Cdc42-palm isoform can still interact with RhoGDI. The reason for such interaction (and as a consequence for greater Cdc42-palm mobility) can be a high basal level of palmitate turnover on Cys\textsuperscript{189}. This can result in the formation of a depalmitoylated Cdc42-palm form that is capable of binding RhoGDI\textsubscript{a}, which in turn will facilitate the membrane trafficking of Cdc42. This is also in line with a recent report by Chandra et al. [47], who demonstrated that binding of the GDI-like solubilizing factor PDE\textsubscript{δ} (phosphodiesterase \delta) to depalmitoylated H-Ras facilitates its diffusion in the cytoplasm and thereby enhances the kinetics of trapping at the plasma membrane. It is noteworthy that in the report by Nishimura and Linder [31] the authors also hypothesize that RhoGDI may operate in a similar way to facilitate repalmitoylation (and thereby the membrane binding) of Cdc42-palm. Alternatively, double-palmitoylated Cdc42-palm can possess a higher affinity for defined membrane subdomains such as mobile membrane rafts with increased lateral mobility [27]. Thus localization of Cdc42-palm in such domains will result in its higher lateral diffusion. The results of the FRAP experiments confirmed this suggestion and showed that Cdc42-palm was significantly higher than that of Cdc42-prenyl isoform. Future studies will be needed to more precisely evaluate the contribution of palmitoylation in increased Cdc42-palm mobility.

Lipidation of Cys\textsuperscript{189} seems also to be necessary for the localization of Cdc42-palm at the plasma membrane, since replacement of this cysteine residue either alone or in combination with Cys\textsuperscript{189} results in weaker transient interaction with the membrane and increased soluble protein homogeneously distributed throughout the whole cell, including the nucleus. Consequently, the mobility of C188A and C188A/C189A mutants is significantly increased in comparison with the wild-type or C189A mutant of Cdc42-palm.

One physiological function of Cdc42 is the activation of transcription factors such as NF-\kappa B (nuclear factor \kappa B), STAT3 (signal transducer and activator of transcription 3) and SRF [9,48,49]. SRF binds to the SRE, and SRE activation is crucial for the transcription of many immediate early genes [50]. Our data demonstrate that Cdc42-palm activates SRE more efficiently than Cdc42-prenyl. This cannot be explained by changes in the intrinsic Cdc42 activation between isoforms, because expression of CA mutants results in similar SRE activation. One possible reason could be the involvement of Cdc42 palmitoylation in trafficking and/or localization into specific membrane subdomains, such as lipid rafts. Protein modification by the covalent attachment of palmitate represents one of the best characterized lipid-raft-targeting signals [51,52]. Accordingly, a number of acylated proteins, including heterotrimeric G-protein \alpha-subunits, Src family kinases and small GTPase H-Ras are resident in the lipid rafts [51,53–55]. Thus palmitoylation-mediated lipid raft localization of Cdc42-palm might result in its sustained interaction with raft-residing downstream effectors such as Pak1 (p21-activated kinase 1), WASP (Wiskott–Aldrich syndrome protein) or p38, which in turn can influence the SRE activity by changes in actin dynamics [16,52]. This is supported by the observation that mutation of palmitoylated Cys\textsuperscript{189} results in a reduction in SRE activation to the level obtained for the Cdc42-prenyl wild-type, even though this mutant is properly localized at the plasma membrane. Also, the substitution of the CIF sequence of Cdc42-palm by the VLL motif from Cdc42-prenyl leads to decreased SRE activation without affecting the plasma membrane localization. Hence it will be interesting to study whether lipidation status influences the subdomain localization and functions of Cdc42 isoforms.
The most prominent function of small GTPases is the regulation of actin cytoskeleton leading to changes in cellular morphology. To study the morphogenic potential of Cdc42 lipidation we performed morphological analysis in N1E cells. Overexpression of both isoforms results in induction of densely packed filopodia. Mutation of Cys188 abolishes the targeting of Cdc42-palm to the plasma membrane. Consequently, formation of densely packed filopodia by this mutant is completely abolished and cannot be rescued even by introduction of CA mutation. This demonstrates that only when Cdc42 is properly associated with the plasma membrane, its nucleotide-bound state becomes the decisive factor for Cdc42-mediated morphological changes.

It has previously been shown that the Cdc42-palmitoyl isoform is expressed in all tissues, whereas Cdc42-palm is expressed only in the brain [11,18]. Quantitative real-time PCR analysis performed in the present study revealed the Cdc42-palmitoyl mRNA to be the dominant species in a range of commonly used laboratory cell lines. Notably, this was also the case for cell lines of neuronal origins, such as PC12, mouse brain neuroblastoma N1E-115 and mouse motor neuron-derived NSC34 cells. Similarly, Cdc42-palmitoyl represents a dominant isoform in the mouse cerebellum. In contrast, the expression level of Cdc42-palm in the hippocampus was approximately 2-fold higher than Cdc42-palmitoyl, suggesting a functional importance of the Cdc42-palm isoform in addition to Cdc42-palmitoyl in this region. It is known that the neuronal Cdc42 activity modulates multiple signalling pathways and plasticity-related mechanisms involved in regulation of spine formation and dendritic morphology [7,56,57]. To explore the exact function of Cdc42 in hippocampal neurons, we constructed AAVs to knockdown both endogenous Cdc42 isoforms and to simultaneously introduce shRNA-resistant Cdc42-palmitoyl or Cdc42-palm isoform into neurons. Our data suggest that both Cdc42 isoforms can be responsible for the formation of dendritic protrusions in hippocampal neurons, because introduction of either Cdc42-palm or Cdc42-palmitoyl was able to rescue the inhibitory effect of anti-Cdc42 shRNA treatment. This is in line with observations by Kang et al. [18], even though these authors demonstrated a significantly stronger effect of Cdc42-palm on the spine formation. Such discrepancies can be explained by the different experimental design: in our experiment we knocked down both endogenous isoforms by siRNA and replaced these with either Cdc42-palm or Cdc42-palmitoyl, whereas in experiments by Kang et al. [18], CA Cdc42 mutants were overexpressed in intact neurons. Detailed analysis of acylation-deficient mutants revealed a pivotal role of Cys188 for the Cdc42-palmitoylated induction of dendritic protrusion. The C189A mutant demonstrated more complex behaviour, whereas the number of LPs was not affected, formation of the SP was significantly reduced. The SPs are thought to be spine precursors [58], suggesting a particular role of Cys188 palmitoylation in Cdc42-mediated spinogenesis. Thus our findings demonstrate that lipidation of Cdc42-palm represents an important regulator of morphogenic signalling in hippocampal neurons. Together with the observation that Cdc42-palm undergoes palmitoylation in human brain, this suggests the importance of Cdc42 lipidation for the growth of new synapses as well as for activity-dependent structural and functional plasticity, including learning and long-term memory in humans.

ACKNOWLEDGMENTS

We are grateful to Ursula Jensen and Dalia Abdel-Galil for expert technical assistance and to Sonal Prasad for proofreading the paper before submission.

FUNDING

These studies were supported by the Deutsche Forschungsgemeinschaft (DFG) [grant numbers P0732 and SFB621/C12 (to E.G.P.)] and "Hochschulintensive Leistungsförderung" (HILF)-Fonds [number 79771006] from Hannover Medical School (to C.C.-W.). M.F. was supported by the Ministry of Education and Science of Russian Federation, Grant for Leading Scientists [number 11.G34.31.001].

AUTHOR CONTRIBUTION

Alexander Wirth, Chen Chen-Wacker, Yao-Wen Wu, Mikhail Filippov and Nataliya Gorinski performed the experiments and analysed the data. Ghanshyam Pandey designed the research and analysed the data. Evgeni Ponimaskin, Chen Chen-Wacker and Alexander Wirth wrote the paper.

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Received 13 June 2013/12 September 2013; accepted 23 September 2013
Published as BJ Immediate Publication 23 September 2013, doi:10.1042/BJ20130788
SUPPLEMENTARY ONLINE DATA
Dual lipidation of the brain-specific Cdc42 isoform regulates its functional properties

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Figure S1 Construction and verification of AAV vectors encoding for shRNA against both Cdc42 isoforms

(A) Schematic presentation of the AAV construct containing shRNA against endogenous Cdc42 along with shRNA-resistant Cdc42 tagged with GFP. H1, cassette for shRNA production; ITR, inverted terminal repeats; Syn, synapsin promoter; WPRE, woodchuck hepatitis virus enhancer.

(B) Relative expression level of endogenous Cdc42-palm and Cdc42-prenyl in hippocampal neurons after infection with AAV containing anti-Cdc42 shRNA was determined using quantitative real-time PCR and ΔΔCt method.

Received 13 June 2013/12 September 2013; accepted 23 September 2013
Published as BJ Immediate Publication 23 September 2013, doi:10.1042/BJ20130788

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