DLC1 interacts with 14-3-3 proteins to inhibit RhoGAP activity and block nucleocytoplasmic shuttling

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Summary

Deleted in liver cancer 1 (DLC1) is a Rho-GTPase-activating protein (GAP) that is downregulated in various tumor types. In vitro, DLC1 specifically inactivates the small GTPases RhoA, RhoB and RhoC through its GAP domain and this appears to contribute to its tumor suppressor function in vivo. Molecular mechanisms that control DLC1 activity have not so far been investigated. Here, we show that phorbol-ester-induced activation of protein kinase C and protein kinase D stimulates association of DLC1 with the phosphoserine/phosphothreonine-binding 14-3-3 adaptor proteins via recognition motifs that involve Ser327 and Ser431. Association with 14-3-3 proteins inhibits DLC1 GAP activity and facilitates signaling by active Rho. We further show that treatment of cells with phorbol ester or coexpression of 14-3-3 proteins, blocks DLC1 nucleocytoplasmic shuttling, probably by masking a previously unrecognized nuclear localization sequence. The binding to 14-3-3 proteins is thus a newly discovered mechanism by which DLC1 activity is regulated and compartmentalized.

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Key words: Rho GTPases, Actin cytoskeleton, Protein kinases C and D, Serine phosphorylation, Tumor suppressor

Introduction

The *DLC1* gene was first isolated as a candidate tumor suppressor gene in primary human hepatocellular carcinoma and loss of expression has subsequently been shown in other tumor types, including colon, breast, prostate and lung (Durkin et al., 2007b). Transfection of *DLC1* cDNA into different carcinoma cell lines lacking *DLC1* expression inhibited cell growth and tumorigenicity in nude mice (Ng et al., 2000; Yuan et al., 2003; Yuan et al., 2004; Zhou et al., 2004). Recently, the structurally related proteins DLC2 and DLC3 were found to be downregulated in various tumor types and their re-expression in cancer cells similarly inhibited proliferation, colony formation and growth in soft agar (Ching et al., 2003; Durkin et al., 2007a).

DLC1 contains a GAP domain specific for the small GTPases RhoA, RhoB and RhoC, and to a lesser extent Cdc42 (Healy et al., 2008; Wong et al., 2003). It further contains an N-terminal sterile alpha motif (SAM) and a StAR-related lipid transfer (START) domain at its C-terminus, whose functions remain to be characterized. The Rho family of GTPases are important regulators of diverse biological responses, including actin cytoskeletal rearrangements, gene transcription, cell cycle regulation, apoptosis and membrane trafficking (Jaffe and Hall, 2005; Ridley, 2006). Rho proteins cycle between a GTP-bound active state, in which they interact with effector proteins by modulating their activity and localization, and an inactive GDP-bound state. Signaling of growth factor receptors and integrins can induce exchange of GDP for GTP on Rho proteins. This activation of Rho proteins is controlled by the guanine nucleotide-exchange factors (GEFs), which promote the release of bound GDP and facilitate GTP binding, and the GAP proteins, which increase the intrinsic GTPase activity of Rho GTPases to accelerate the return to the inactive state. Activation of Rho is known to induce the assembly of actin stress fibers and focal adhesions, whereas Cdc42 promotes the formation of specialized membrane protrusions called filopodia (Jaffe and Hall, 2005; Ridley, 2006). Indeed, microinjection of p122, the rat homolog of DLC1, suppressed the formation of LPA-induced stress fibers and focal adhesions (Sekimata et al., 1999). Furthermore, stable expression of human DLC1 in hepatocellular and breast carcinoma cell lines was shown to reduce cell motility and invasiveness, consistent with the inhibition of Rho signaling (Goodison et al., 2005; Wong et al., 2005). Interestingly, stable expression of an inactive form of DLC1 produced in a non-small cell lung cancer cell line was recently found to partially suppress anchorage independent growth and invasion in vitro, suggesting that the anti-tumor function of DLC1 may also be determined by GAP-independent activities (Healy et al., 2008).

The human genome is estimated to encode up to 80 RhoGAPs, suggesting a necessity for very tight temporal and spatial regulation of the 20 known Rho GTPase substrates (Moon and Zheng, 2003). Regulation of RhoGAP proteins is achieved by several mechanisms, such as protein or lipid interaction and post-translational modification (Bernards and Settleman, 2005). Although *DLC1* expression has been studied at the transcriptional level, little is known about its regulation at the protein level. Rat DLC1 was recently shown to be phosphorylated by PKB/Akt and ribosomal S6 kinase (RSK) on Ser322, corresponding to Ser329 in the human protein; however, the significance of this phosphorylation remains to be elucidated (Hers et al., 2006).

Scansite analysis of the DLC1 protein sequence at high stringency revealed the presence of a putative 14-3-3 binding motif conserved

amongst different species (TRTRS327LS in human DLC1). Members of the 14-3-3 protein family are ubiquitously expressed small acidic proteins with a molecular mass of approximately 30 kDa. Seven highly conserved 14-3-3 protein isoforms exist in mammals (β, γ, ζ, σ, τ, ε, η), which form homo- and heterodimers (Bridges and Moorhead, 2004; Dougherty and Morrison, 2004). Generally, 14-3-3 proteins function as adaptors that bind to their target proteins in a phosphorylation-dependent manner. Consensus motifs for binding are RSxpSxP and RxxxpSxP, with phosphothreonine capable of replacing phosphoserine (Yaffe et al., 1997). Many target proteins do not contain sequences that conform precisely to these motifs, and in some cases interaction has been shown to be independent of phosphorylation. The crystal structure of 14-3-3 protein dimers revealed that each subunit can independently bind one discrete phosphoserine- or phosphothreonine-containing ligand. Each dimer therefore contains two binding pockets and as a result can interact with two motifs simultaneously, located either on a single target or on separate binding partners. Binding of 14-3-3 proteins often sequesters the target protein in a particular subcellular compartment and the release of 14-3-3 proteins then allows the target to relocate. This relocation is often due to the exposure of an intrinsic subcellular targeting sequence masked by the 14-3-3 protein dimer. Binding of 14-3-3 proteins can also induce conformational changes of the target protein or may have a scaffolding function (Bridges and Moorhead, 2004; Dougherty and Morrison, 2004).

Here we show that DLC1 interacts with 14-3-3 proteins in a phosphorylation-dependent manner, requiring phosphorylation of Ser327 and Ser431. These serines lie within consensus motifs for the protein kinase D (PKD) family of serine/threonine kinases. Activation of PKD requires the recruitment to membranes through diacylglycerol (DAG) binding and involves phosphorylation of its activation loop by novel PKCs (Rykx et al., 2003; Wang, 2006). Thus, treatment of cells with phorbol ester, a DAG analog, stimulated DLC1 phosphorylation and association with 14-3-3 proteins. This interaction was found to suppress DLC1 GAP activity, preventing DLC1-mediated stimulation of Rho-GTP hydrolysis as determined with a RhoA biosensor, and enabling downstream signaling, as shown with serum response factor (SRF)-dependent reporter assays. Accordingly, a DLC1 S327/431A mutant was more active than the wild-type protein in inhibiting cell growth. DLC1 is further revealed to undergo rapid nucleocytoplasmic shuttling, which was inhibited by phorbol-ester-induced phosphorylation and 14-3-3 binding, most likely by masking a novel NLS located adjacent to the Ser431 phosphorylation site.

Results

DLC1 specifically interacts with 14-3-3 proteins

To investigate DLC1 interaction with 14-3-3 proteins, all seven mammalian isoforms were purified as GST fusions from *E. coli* and used in pull-down assays with whole-cell extracts of HEK293T cells transiently expressing Myc-tagged DLC1. Fig. 1A shows that DLC1 associates with all 14-3-3 isoforms with varying affinity except 14-3-3σ, and does not interact with GST alone. 14-3-3 proteins commonly bind target proteins via a conserved amphipathic groove comprising a cluster of basic amino acids. To test whether the interaction with DLC1 was mediated by this region, we made use of a R56/60A 14-3-3τ mutant protein previously shown to be deficient in target protein binding (Hausser et al., 2006; Xing et al., 2000). DLC1 did not bind to the R56/60A mutant in a pull-down assay (Fig. 1B), confirming that association involved the 14-3-3 binding groove. To identify the region in DLC1 responsible for 14-3-3 binding, truncation mutants were generated, in which the N-terminal SAM (ΔSAM) or the C-terminal START domain (ΔSTART) were deleted (Fig. 1C). Deletion of neither domain affected 14-3-3 binding (Fig. 1D). However, the C-terminus of the protein comprising the RhoGAP and START domains (Fig. 1C) (ΔN) failed to interact with GST-14-3-3β, suggesting that DLC1 interaction with 14-3-3 proteins is mediated by the linker region (residues 79-625) connecting the SAM and RhoGAP domains.

The interaction of DLC1 with 14-3-3 proteins was further analyzed by coimmunoprecipitation experiments. Myc-tagged

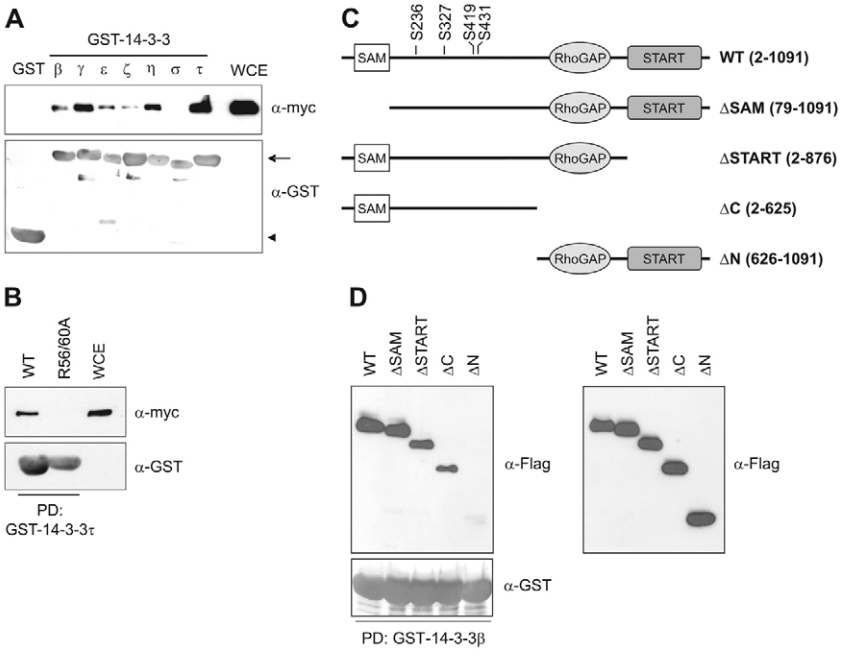

Fig. 1. Interaction of DLC1 with recombinant GST-14-3-3 proteins. (A) HEK293T cells were transiently transfected with a plasmid encoding Myc-tagged DLC1. Whole-cell extracts (WCE) were incubated with glutathione beads coupled to the indicated GST–14-3-3 isoforms, or GST alone, and bound proteins were separated by SDS-PAGE. DLC1 was detected by western blotting with a Myc-specific antibody (top panel). The integrity of GST (marked with an arrowhead) and GST-14-3-3 isoforms (marked with an arrow) was verified by probing the membrane with GST-specific antibody. (B) Whole-cell extracts of HEK293T cells transiently expressing Myc-DLC1 were subjected to a pull-down (PD) with wild-type (WT) or R56/60A GST-14-3-3τ, and bound proteins were analyzed as described in A. (C) Schematic representation of DLC1 truncation mutants and putative PKD phosphorylation sites. (D) Whole-cell extracts of HEK293T cells transiently expressing the indicated Flag-tagged DLC1 mutants were subjected to a pull-down with GST-14-3-3β and bound proteins were analyzed with Flag-specific antibody as described in A. Expression of the different DLC1 variants was verified by immunoblotting of whole-cell extracts with Flag-specific antibody (right panel).

DLC1 was coexpressed with either Glu-Glu (EE)-tagged 14-3-3ζ or γ, or HA-tagged 14-3-3τ in HEK293T cells. 14-3-3 proteins were then immunoprecipitated from whole-cell extracts using the respective HA- or EE-specific antibodies. DLC1 was found to coprecipitate with 14-3-3τ and γ, but did not interact with 14-3-3ζ under these conditions (Fig. 2A), which correlates with the weak interaction seen with the ζ isoform in the pull-down assay. The same results were obtained by first precipitating Myc-DLC1 and subsequent detection of 14-3-3 isoforms (Fig. 2B). Thus, DLC1 also interacts with 14-3-3 proteins expressed in eukaryotic cells, confirming the results obtained in the pull-down assays. Finally, the association of DLC1 with endogenous 14-3-3 proteins was investigated. HEK293T cells were transiently transfected with Myc-tagged DLC1 and whole-cell extracts were incubated with either 14-3-3-specific antibody that recognizes all 14-3-3 isoforms or a control IgG. DLC1 was detected in the immunoprecipitate of 14-3-3 proteins (Fig. 2C), proving that DLC1 also associates with endogenous 14-3-3 proteins.

Phorbol ester stimulation enhances DLC1 association with 14-3-3 proteins

In most cases, recognition of target proteins by 14-3-3 proteins is phosphorylation dependent and thus controlled by the kinases and phosphatases that modulate the phosphorylation state of the target protein. To first analyze whether the interaction of DLC1 with 14-3-3 proteins was dependent upon phosphorylation, we treated HEK293T cells transiently expressing Myc-DLC1 with the serine/threonine phosphatase inhibitor okadaic acid. Cells were lysed and subjected to pull-down assays with GST-14-3-3τ. Compared with the DMSO control, okadaic acid treatment of cells strongly enhanced 14-3-3τ binding to DLC1 after 2 and 4 hours (Fig. 3A, top panel). Additionally, the DLC1 protein displayed lower electrophoretic mobility after okadaic acid treatment (Fig. 3A, bottom panel), suggestive of increased phosphorylation. This substantial phosphoshift indicates that DLC1 is a phosphoprotein that might be phosphorylated on several sites. Okadaic acid is an effective inhibitor of protein phosphatase 2A (PP2A) and the less abundant protein phosphatase-4 and -5, but has no detectable inhibitory effect on protein phosphatase-1 or other major serine/threonine phosphatases (Favre et al., 1997). Because DLC1 phosphorylation increased after okadaic acid treatment, it is thus likely that DLC1 is a target for PP2A.

To further characterize the interaction of DLC1 with 14-3-3 proteins, we aimed to identify the signaling pathways involved in the regulation of this interaction. Phorbol-12,13-dibutyrate (PDBu), an analog of DAG, was found to increase association of DLC1 with GST-14-3-3τ as early as 15 minutes after stimulation of cells, with elevated binding observed for up to 2 hours (Fig. 3B). The main cellular kinases that directly bind and are activated by phorbol esters are the classical PKCs (α, β, γ), the novel PKCs (δ, η, ε, θ), and the PKD family members PKD1/PKCμ, PKD2 and PKD3/PKCν (Griner and Kazanietz, 2007). To investigate the potential involvement of these kinases in DLC1 phosphorylation and 14-3-3 binding, we tested the ability of different pharmacological inhibitors to block PDBu-induced 14-3-3 binding to DLC1. Staurosporine is a non-specific kinase inhibitor that inhibits PKCs and PKD, the more selective compound Gö6983 inhibits classical and novel PKCs, and Gö6976 inhibits both classical PKCs and PKD. Pretreatment of cells with these inhibitors blocked PDBu-induced 14-3-3τ binding to DLC1 (Fig. 3C), whereas pharmacological inhibition of other AGC kinases, such as PKA (with H89) and PKB/Akt (by blocking PI3K with LY294002), had no effect (data not shown). Since novel PKC-mediated phosphorylation of the PKD activation loop is required for PKD kinase activity, inhibition of novel PKCs is expected to impinge on PKD substrate phosphorylation. Finally, we verified that endogenous DLC1 also associates with 14-3-3 proteins in response to phorbol ester stimulation by performing a GST-14-3-3τ pull-down of DLC1 from lysates of MDAMB231

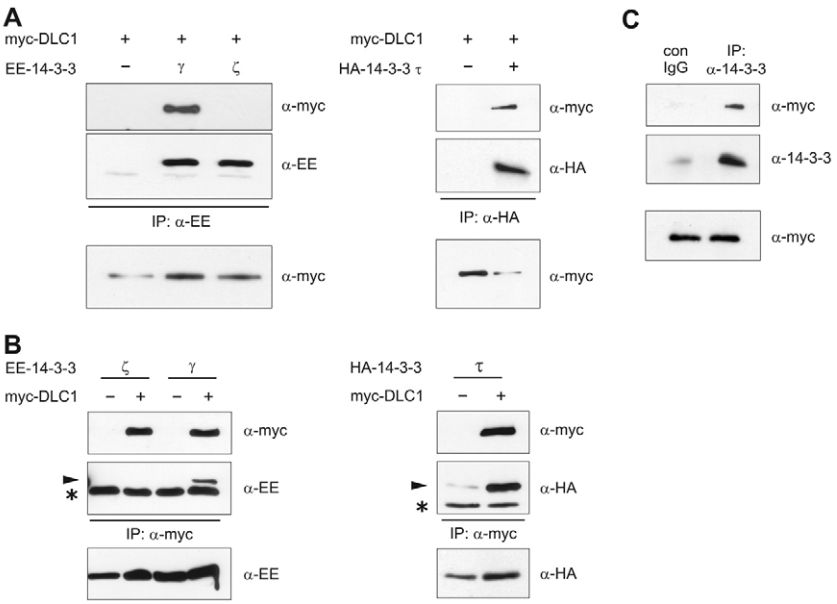

Fig. 2. Coimmunoprecipitation of DLC1 with 14-3-3 proteins. (A) HEK293T cells were transiently transfected with Myc-tagged DLC1 and empty vector (–), EE-tagged 14-3-3γ or 14-3-3ζ or HA-tagged 14-3-3τ expression vectors. 14-3-3 isoforms were immunoprecipitated from whole-cell extracts with EE- and HA-specific antibodies, respectively, and immune complexes were separated by SDS-PAGE. Coprecipitated DLC1 was detected by western blotting with Myc-specific antibody (top panels). Immunoprecipitation of 14-3-3 isoforms was verified by probing the membrane with EE- and HA-specific antibodies, respectively (middle panels), and expression of DLC1 was verified by immunoblotting of whole-cell extracts with Myc-specific antibody (bottom panels). (B) HEK293T cells were transiently transfected with expression vectors encoding EE-tagged 14-3-3γ or 14-3-3ζ, or HA-tagged 14-3-3τ, and empty vector or Myc-tagged DLC1. DLC1 was immunoprecipitated from whole-cell extracts with Myc-specific antibody and immune complexes were separated by SDS-PAGE. Coprecipitated 14-3-3 isoforms were detected by western blotting with EE- and HA-specific antibodies, respectively (middle panels, indicated with arrowheads). IgG chains are indicated with asterisks. Immunoprecipitation of DLC1 was verified with Myc-specific antibody (top panels), and expression of 14-3-3 isoforms was verified by immunoblotting of whole-cell extracts with EE- and HA-specific antibodies (bottom panels). (C) HEK293T cells transiently expressing Myc-tagged DLC1 were lysed and 14-3-3 proteins were immunoprecipitated from whole-cell extracts with 14-3-3-specific rabbit pAb. An unrelated rabbit pAb was used as a control (con IgG). Immune complexes were separated by SDS-PAGE. Coprecipitated DLC1 was detected by western blotting with Myc-specific antibody (top panel). Immunoprecipitation of 14-3-3 proteins were verified by probing the membrane with 14-3-3-specific mouse mAb (middle panel). Expression of DLC1 was verified by immunoblotting of whole-cell extracts with Myc-specific antibody (bottom panel).

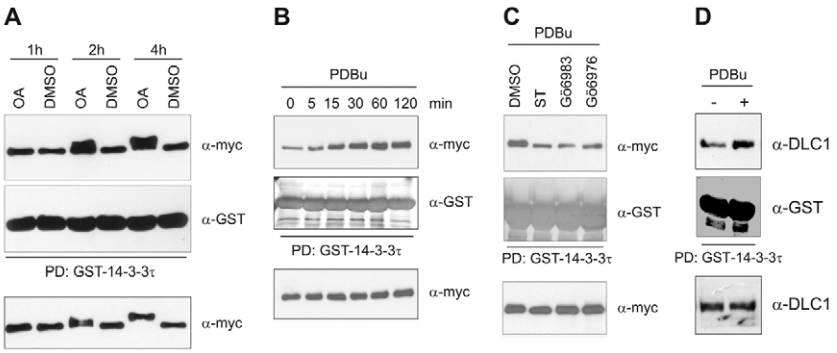

Fig. 3. Phosphorylation-dependent interaction of DLC1 with 14-3-3 proteins. HEK293T cells were transiently transfected with a Myc-DLC1 expression vector and treated with (A) 100 nM okadaic acid or solvent (DMSO), or (B) 100 nM PDBu for the indicated times prior to lysis. (C) Cells were incubated with 1 μM staurosporine (ST), 5 μM Gö6983, 5 μM Gö6976 or solvent (DMSO) for 90 minutes before stimulation with 1 μM PDBu for 15 minutes. (D) MDAMB231 cells were left untreated or treated with 1 μM PDBu for 15 minutes prior to lysis. Whole-cell extracts were subjected to a pull-down with GST-14-3-3τ beads and bound proteins were separated by SDS-PAGE. DLC1 was detected with Myc-specific antibody in A-C and DLC1-specific antibody in D (top panels). The integrity of recombinant GST-14-3-3τ was verified by probing the membrane with GST-specific antibody (middle panels), and expression of DLC1 was verified by immunoblotting of whole-cell extracts with Myc-specific antibody in A-C and DLC1-specific antibody in D (bottom panels).

breast cancer cells (Fig. 3D). Together, these results suggest that activation of phorbol ester-responsive PKCs and/or PKD regulate DLC1 association with 14-3-3 proteins.

DLC1 phosphorylation on serines 327 and 431 is required for interaction with 14-3-3 proteins

In silico analysis of DLC1 with Motif Scan predicted several potential 14-3-3 binding sites, with Ser327 fulfilling high stringency criteria (Obenauer et al., 2003). Interestingly, Ser327 lies within an optimal consensus sequence for PKD kinases (L/I/VxRxxS/T), determined by an arginine in the –3 and a leucine, isoleucine or valine in the –5 position relative to the serine or threonine to be phosphorylated (Nishikawa et al., 1997). Intriguingly, PKD-mediated phosphorylation of substrates such as phosphatidylinositol 4-kinase IIIβ, histone deacetylase 5 and the Ras effector RIN1 has been described to generate 14-3-3 recognition motifs (Hausser et al., 2006; Vega et al., 2004; Wang et al., 2002). We therefore coexpressed GFP-tagged PKD1 with Flag-DLC1 in HEK293T cells and analyzed DLC1 binding to GST-14-3-3τ with pull-down assays. Coexpression of PKD1 was

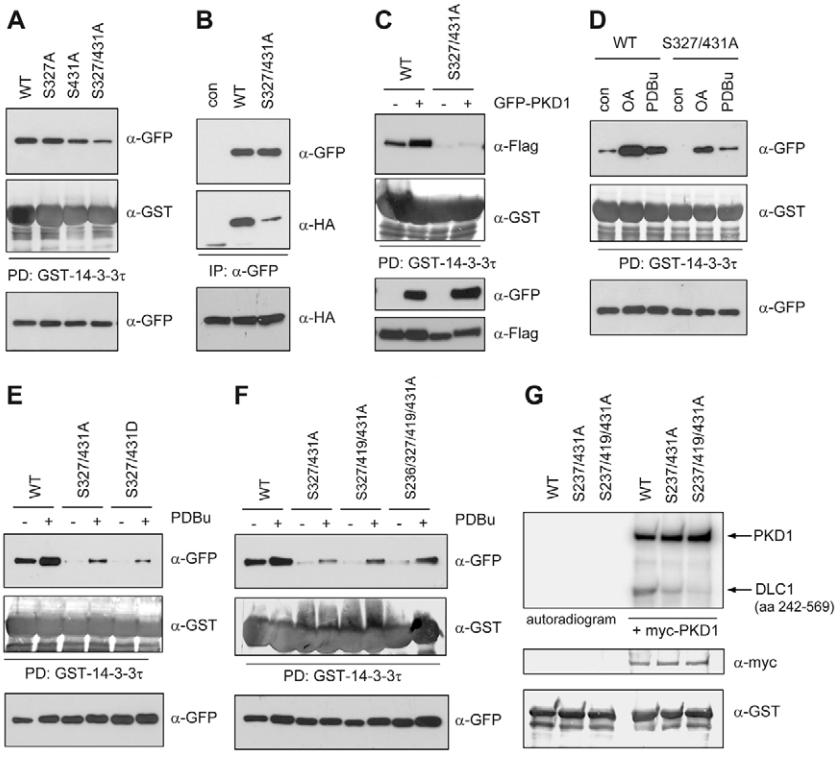

Fig. 4. DLC1 interaction with 14-3-3 proteins requires phosphorylation of Ser327 and Ser431. HEK293T cells were transiently transfected with GFP-tagged DLC1 WT, S327A, S431A, or S327/431A expression vectors. Whole-cell extracts were subjected to a pull-down with GST-14-3-3τ beads and bound proteins were separated by SDS-PAGE. DLC1 variants were detected with a GFP-specific antibody (top panel). The integrity of recombinant GST-14-3-3τ was verified by probing the membrane with GST-specific antibody (middle panel), and expression of DLC1 variants was verified by immunoblotting of whole-cell extracts with GFP-specific antibody (bottom panel). (B) An HA-tagged 14-3-3τ expression vector was transiently transfected into HEK293T cells along with DLC1 WT, S327/431A or empty vector (con). DLC1 variants were immunoprecipitated from whole-cell extracts with GFP-specific antibody and immune complexes were separated by SDS-PAGE. Coprecipitated 14-3-3τ was detected by western blotting with HA-specific antibody (middle panel). Immunoprecipitation of DLC1 variants was verified by probing the membrane with GFP-specific antibody (top panel). Equal expression of 14-3-3τ was verified by immunoblotting of whole-cell extracts with HA-specific antibody (bottom panel). (C) HEK293T cells were transiently transfected with expression plasmids encoding Flag-tagged DLC1 WT or S327/431 and empty vector (–) or GFP-tagged PKD1 (+), respectively. Whole-cell extracts were subjected to GST-14-3-3τ pull-downs and analyzed as described in A. Expression of PKD1 and DLC1 variants was verified by immunoblotting of whole-cell extracts with GFP- and Flag-specific antibodies, respectively (bottom panels). (D) HEK293T transiently expressing GFP-DLC1 WT or S327/431A were treated left untreated or treated with 100 nM okadaic acid for 2 hours or 1 μM PDBu for 15 minutes. Whole-cell extracts were subjected to GST-14-3-3τ pull-downs and analyzed as described in A. (E) HEK293T cells transiently expressing GFP-tagged DLC1 WT, S327/431A or S327/431D were left untreated (–) or treated with 1 μM PDBu (+) for 15 minutes. Whole-cell extracts were subjected to GST-14-3-3τ pull-downs and analyzed as described in A. (F) HEK293T cells transiently expressing GFP-tagged DLC1 WT, S327/431A, S327/419/431A, or S236/327/419/431A were left untreated (–) or treated with 1 μM PDBu (+) for 15 minutes. Whole-cell extracts were subjected to GST-14-3-3τ pull-downs and analyzed as described in A. (G) Recombinant GST-DLC1(aa242-569) proteins were incubated in kinase buffer containing [γ-^{32}P]ATP in the absence (left lanes) or presence of purified Myc-tagged PKD1 (right lanes). Proteins were separated by SDS-PAGE and transferred to membrane. Incorporation of radioactive phosphate was analyzed using a PhosphoImager (top panel), followed by immunoblotting with GST- and Myc-specific antibodies to verify equal loading (bottom panels). The GST-DLC1 fusion proteins and Myc-PKD1 are indicated with arrows.

indeed found to enhance DLC1 interaction with 14-3-3τ (Fig. 4C, lanes 1-2). To test whether Ser327 in DLC1 was essential for association with 14-3-3 proteins, we exchanged this serine for an alanine by site-directed mutagenesis. However, no obvious difference in the interaction with GST-14-3-3τ was observed between wild-type DLC1 and the S327A mutant protein (Fig. 4A, lanes 1-2). The linker region of DLC1 identified to mediate 14-3-3 binding contains three additional serines at positions 236, 419 and 431 that match PKD substrate site criteria (see Fig. 1C). Owing to the dimeric nature of 14-3-3 proteins, target protein binding often involves more than one phosphorylation site. We therefore generated combinatorial DLC1 phosphorylation site mutants, because individual mutation of these sites failed to abolish DLC1 binding to 14-3-3 proteins. Interestingly, a DLC1 double mutant, in which Ser327 and Ser431 were mutated to alanines, displayed reduced 14-3-3 binding in both pull-down assays and coimmunoprecipitations (Fig. 4A,B). Additional mutation of Ser236 and Ser419 did not reduce 14-3-3 binding any further (Fig. 4F), suggesting that Ser327 and Ser431 in DLC1 are the only sites that confer 14-3-3 binding downstream of PKD. This is supported by the finding that PKD1 coexpression with the DLC1 S327/431A mutant no longer enhanced 14-3-3 binding as it did when coexpressed with wild-type DLC1 (Fig. 4C). The residual 14-3-3 binding to the DLC1 S327/431A mutant (observed in longer exposures) and higher-order mutants could still be enhanced by okadaic acid or PDBu treatment, suggesting at least one additional PKD-independent phosphorylation site that contributes to 14-3-3 binding (Fig. 4D,F). We attempted to generate a DLC1 variant that constitutively binds 14-3-3 proteins by exchanging Ser327 and Ser431 to glutamic acid. However, this DLC1 S327/431D mutant was indistinguishable from the serine-to-alanine double-exchange mutant in terms of 14-3-3 binding (Fig. 4E), indicating that introduction of negative charge is not sufficient to mimic a phosphorylated 14-3-3 recognition motif.

To provide direct evidence that PKD is capable of phosphorylating Ser327 and Ser431 in DLC1 we fused a fragment spanning these sites (residues 242-569) to GST and subjected the recombinant fusion protein to an in vitro kinase assay with purified PKD1. This fusion protein was phosphorylated only in the presence of PKD1 (Fig. 4G). A fusion protein in which Ser327 and Ser431 were mutated to alanines incorporated significantly lower amounts of radiolabeled phosphate, proving that these serines are genuine PKD phosphorylation sites (Fig. 4G). No phosphorylation was observed when Ser419, which also matches a PKD consensus sequence, was additionally mutated (Fig. 4G). However, as shown in Fig. 4F, this site does not contribute to 14-3-3 binding.

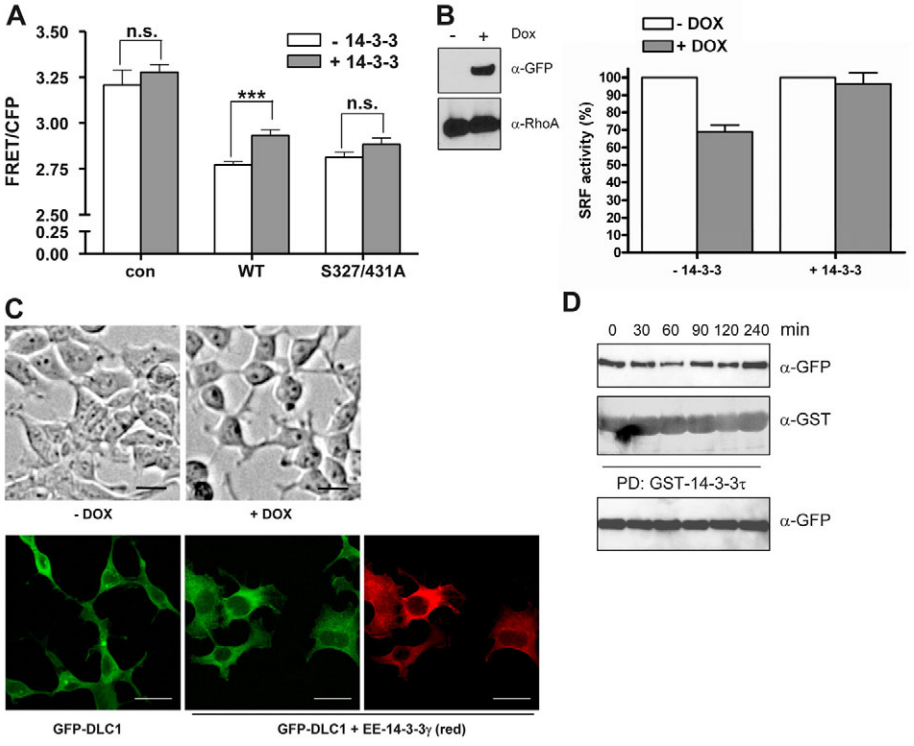

Fig. 5. Binding of 14-3-3 proteins inhibits DLC1 RhoGAP activity. (A) HEK293T cells were transiently transfected with expression vectors encoding Raichu-RhoA (con) along with Flag-DLC1 WT or S327/431A, and HA-14-3-3τ, where indicated. The emission ratio of Raichu-RhoA was determined by measuring YFP (FRET) and CFP fluorescence (excitation 433 nm) in cell lysates. Equal expression of 14-3-3τ and DLC1 proteins was verified by immunoblotting of lysates with HA- and Flag-specific antibodies, respectively (not shown). The mean of three independent experiments performed with triplicate samples is shown; error bars represent s.e.m. Results were statistically significant for the wild-type protein (two-tailed unpaired *t*-test, P=0.0006); no significant difference was observed for the control and the S327/431A mutant protein (NS, P>0.05). (B) HEK293 Flp-In-DLC1 cells were left untreated (–) or treated with 10 ng/ml doxycycline (+) overnight to induce GFP-DLC1 expression. Whole-cell extracts were separated by SDS-PAGE and analyzed by western blotting with GFP-specific (top panel) and RhoA-specific antibodies (bottom panel). For SRF reporter assays, Flp-In-DLC1 cells were transiently transfected with the 3DA.Luc reporter, pTK-Renilla and HA-14-3-3τ or empty vector. Cells were left untreated (–DOX) or treated with 10 ng/ml doxycycline (+DOX) for 4 hours, followed by PDBu stimulation (100 nM) for additional 4 hours. Firefly luciferase activity in cell lysates was determined and normalized by *Renilla* luciferase activity. Fold induction after PDBu stimulation was calculated and values for uninduced cells (–DOX) were set to 100%. Expression of DLC1 and 14-3-3 proteins was verified by immunoblotting of lysates with GFP- and HA-specific antibodies, respectively (not shown). The mean of three independent experiments performed with triplicate samples is shown, error bars represent s.e.m. (C) Flp-In-DLC1 cells were left untreated or treated with 10 ng/ml doxycycline over night and photographed with a Leitz DM IRB microscope equipped with a NPLAN 10/0.25 PH1 objective (Leica) and an AxioCam MRc camera (Zeiss) (left panels). Flp-In-DLC1 cells were transiently transfected with empty vector or EE-14-3-3γ expression plasmid, followed by induction of GFP-DLC1 expression with 10 ng/ml doxycycline overnight. Cells were fixed and stained with EE-specific antibody (red) (right panels). Stacks of several confocal sections are shown. Scale bars: 20 μm. (D) Flp-In-DLC1 cells were treated with 10 ng/ml doxycycline overnight, trypsinized and kept in suspension for 1 hour. Cells were then plated onto collagen-coated dishes for the indicated times and lysed. Whole-cell extracts were subjected to a pull-down with GST-14-3-3τ beads and bound proteins were separated by SDS-PAGE. DLC1 was detected with GFP-specific antibody (top panel). The integrity of recombinant GST-14-3-3τ was verified by probing the membrane with GST-specific antibody (middle panel), and expression of DLC1 was verified by immunoblotting of whole-cell extracts with GFP-specific antibody (bottom panel).

Binding of 14-3-3 proteins blocks DLC1 RhoGAP function

We next sought to address the functional consequences of 14-3-3 binding to DLC1. DLC1 is a GAP protein reported to enhance GTP hydrolysis rates of RhoA, RhoB and RhoC (Healy et al., 2008; Wong et al., 2003). To study how 14-3-3 binding may affect DLC1-mediated Rho inactivation we made use of a genetically encoded fluorescence resonance energy transfer (FRET)-based RhoA biosensor, termed Raichu-RhoA (Yoshizaki et al., 2003). This sensor consists of full-length RhoA, the Rho-binding domain (RBD) of the effector PKN, which specifically binds Rho-GTP, and the fluorescence donor-acceptor pair CFP and YFP. Upon activation by GTP loading, the RBD binds RhoA, modifying the orientation of the fusion protein and allowing FRET to occur. RhoA activation is approximately proportional to the ratio of FRET/CFP emission. As expected, transient expression of Raichu-RhoA together with DLC1 in HEK293T cells led to a decreased emission ratio measured in cellular lysates, indicating stimulation of RhoA-GTP hydrolysis (Fig. 5A). This was partially reversed upon coexpression of 14-3-3τ, suggesting that association with 14-3-3 proteins inactivates DLC1 GAP function. By contrast, coexpression of 14-3-3τ had no significant effect on the GAP activity of DLC1 S327/431A in this assay (Fig. 5A).

To address how 14-3-3 binding to DLC1 impacts on endogenous Rho signalling, we analyzed the ability of DLC1 to block SRF-dependent transcription, which is known to require functional Rho (Posern and Treisman, 2006). Here we used Flp-In T-Rex HEK293 cells, which stably express the Tet repressor and contain a single genomic Flp recombination target site to generate an inducible *DLC1*-expressing line by Flp-recombinase-mediated integration of a GFP-tagged *DLC1* cDNA under the control of tetracycline-regulated promoter. In these stable Flp-In HEK293 DLC1 cells, expression of GFP-DLC1 was tightly controlled, with no expression observed in the absence of doxycycline (Fig. 5B, top panel). Cellular RhoA levels were not affected by doxycycline-induced DLC1 expression (Fig. 5B, bottom panel). These cells were transfected with an SRF-responsive luciferase reporter along with empty vector or a 14-3-3τ expression plasmid, serum-starved and doxycycline was added to induce DLC1 expression. Cells were then stimulated with PDBu, which activates SRF-mediated transcription and simultaneously induces DLC1 phosphorylation. DLC1 expression was found to inhibit PDBu-induced SRF-dependent transcription by approximately 30% compared with the uninduced control (Fig. 5B). Coexpression of 14-3-3τ almost completely abrogated the inhibitory effect of DLC1 on SRF-mediated gene expression (Fig. 5B), confirming the results obtained with the exogenous RhoA biosensor.

Overexpression of DLC1 has been shown to cause cell detachment associated with the disassembly of stress fibers and focal adhesions, which require active Rho for establishment and maintenance (Sekimata et al., 1999; Wong et al., 2005). In line with these observations, Flp-In HEK293 cells lost their spread appearance and presented with a rounded cell body and long spindle-shaped extensions when DLC1 expression was induced (Fig. 5C). Transfection of cells with an expression plasmid encoding 14-3-3γ prior to DLC1 induction rescued this phenotype (Fig. 5C). RhoA activity is transiently inhibited during the course of cell adhesion and spreading (Arthur et al., 2000; Ren et al., 1999). To investigate the potential dynamic regulation of DLC1 by integrin engagement, we performed GST-14-3-3τ pull-down assays with lysates from Flp-In HEK293 cells induced to express *DLC1* and plated onto collagen-coated dishes for different time periods (Fig. 5D). In suspended cells, DLC1 was found to associate with 14-3-3τ. Upon plating, DLC1 interaction with GST-14-3-3τ initially decreased and then increased again at later times (Fig. 5D), indicating an inverse relationship between DLC1 and RhoA activation kinetics in adhering cells.

Ectopic expression of DLC1 has been shown to inhibit cell proliferation (Ng et al., 2000; Yuan et al., 2003; Yuan et al., 2004; Zhou et al., 2004). We therefore stably expressed Flag-tagged wild-type DLC1 and the S327/431A mutant in MCF7 cells, which express low levels of endogenous DLC1 (Fig. 6A), and analyzed their impact on cell proliferation. Compared to the wild-type protein, the DLC1 mutant was more active in inhibiting cell growth when assessed by MTT assays (Fig. 6B). To verify that DLC1 was subject to the same molecular regulation in these cells, we performed pull-down assays with GST-14-3-3τ. As seen in HEK293 cells, the wild-type protein was readily precipitated from MCF7 cell lysates in a PDBu-inducible fashion, whereas interaction was barely visible in the case of the S327/431A mutant protein (Fig. 6C). Wild-type DLC1 further

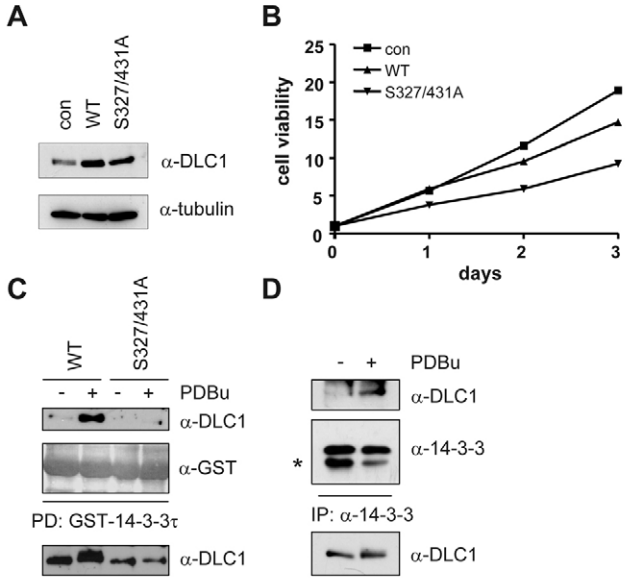

Fig. 6. Stable expression of DLC1 variants in MCF7 cells inhibits proliferation. (A) Stable expression of DLC1 variants in MCF7 cells was verified by immunoblotting of whole-cell extracts using a DLC1-specific antibody (top panel). Equal loading was confirmed by probing the membrane with tubulin-specific antibody (bottom panel). (B) Proliferation of MCF7 cells stably overexpressing DLC1 WT and S327/431A, respectively, and vector control cells was measured by MTT assay. Data are normalized to absorbance at day 0. (C) MCF7 DLC1 WT and S327/431A cells were left untreated or treated with 1 μM PDBu for 15 minutes prior to lysis. Whole-cell extracts were subjected to a pull-down with GST-14-3-3τ beads and bound proteins were separated by SDS-PAGE. DLC1 was detected with DLC1-specific antibody (top panel). The integrity of recombinant GST-14-3-3τ was verified by probing the membrane with GST-specific antibody (middle panel), and expression of DLC1 was verified by immunoblotting of whole-cell extracts with DLC1-specific antibody (bottom panel). (D) MCF7 DLC1 WT cells were left untreated or treated with 1 μM PDBu prior to lysis. 14-3-3 proteins were immunoprecipitated from whole-cell extracts with 14-3-3-specific rabbit pAb. Immune complexes were separated by SDS-PAGE. Coprecipitated DLC1 was detected by Western blotting with DLC1-specific antibody (top panel). Immunoprecipitation of 14-3-3 proteins were verified by probing the membrane with 14-3-3-specific rabbit pAb (middle panel; IgG chains are indicated with an asterisk). Expression of DLC1 was verified by immunoblotting of whole-cell extracts with DLC1-specific antibody (bottom panel).

coimmunoprecipitated with endogenous 14-3-3 proteins upon PDBu stimulation of MCF7-DLC1 cells (Fig. 6D). Taken together, these data suggest that the phosphorylation-dependent interaction with 14-3-3 proteins negatively regulates DLC1 function, by preventing inactivation of Rho signaling.

Binding of 14-3-3 proteins inhibits DLC1 nuclear import by masking a novel NLS

14-3-3 binding often affects cellular localization of protein partners by sequestration. We noted that Ser431 in DLC1 lies in close proximity to a potential NLS that follows the 'pat7' rule. This pattern starts with a proline and is followed within three residues by a basic segment containing three arginine or lysine residues out of four (Fig. 7A, residues 423-429, underlined). The basic cluster at this position may also be part of a bipartite NLS, which is characterized by two basic residues, a ten-residue spacer, and another basic region consisting of at least three basic residues out of five (Fig. 7A, highlighted in bold) (Nakai and Horton, 1999). These basic residues are recognized by importin α proteins, which bind cargo to be transported to the nucleus. To first investigate whether DLC1 shuttles through the nucleus, we expressed GFP-tagged DLC1 in MCF7 cells, followed by treatment of cells with leptomycin B (LMB), an antifungal antibiotic that inhibits Crm1-dependent nuclear export. In untreated cells, DLC1 was evenly distributed in the cytoplasm, without any nuclear localization detectable under these steady-state conditions (Fig. 7B, left panel). However, after treatment of cells with LMB for 2 hours, DLC1 was predominantly found in the nucleus (Fig. 7B, right panel), indicating that DLC1 molecules undergo continuous nuclear import and export. To determine whether the potential mono- or bipartite NLS neighboring Ser431 was responsible for DLC1 nuclear import, we exchanged the Arg428 and Arg429 with glycine residues and analyzed protein localization before and after LMB treatment. In the absence of LMB, the DLC1 R428/429 mutant was indistinguishable from the wild-type protein (data not shown). However, after 2 hours of LMB treatment, the DLC1 R428/429 mutant was homogenously distributed in both compartments (Fig. 7C, left panel). Less than 20% of cells displayed accumulation of the DLC1 R428/429 mutant protein in the nucleus, compared with ~80% of cells expressing the wild-type protein (Fig. 7D). Mutation of Arg415 and Arg416 had a negligible effect on LMB-induced DLC1 nuclear accumulation, indicating that these residues are unlikely to be part of a bipartite NLS (Fig. 7D). To rule out any contribution of this first basic cluster, we generated a quadruple DLC1 R415/416/428/429G mutant. This mutant demonstrated nuclear import kinetics identical to the R428/429G mutant (Fig. 7D), suggesting that the second basic cluster is sufficient for the generation a functional NLS. Since the R428/429G mutant displayed slower import kinetics but was not excluded from the nucleus completely, other motifs that contribute to DLC1 nuclear import are likely to exist. In the DLC1 N-terminus three additional potential NLS were found using the Psort prediction program. However, mutation of any of these basic clusters did not affect LMB-induced nuclear accumulation of DLC1. It is possible that these sequences assist in nuclear entry and only when mutated simultaneously will the protein escape recognition and be trapped in the cytoplasm.

We next tested whether the phosphorylation status was involved in the regulation of DLC1 nucleocytoplasmic shuttling. To this end, cells expressing GFP-DLC1 were treated with PDBu for 15 minutes before LMB administration for 2 hours. As shown in Fig. 7C (right panel), phorbol ester stimulation prevented LMB-induced nuclear accumulation of DLC1, raising the possibility that DLC1 phosphorylation and 14-3-3 binding may interfere with NLS function. In agreement with this hypothesis, the DLC1 S327/431A mutant identified to be severely impaired in 14-3-3 binding accumulated more rapidly in the nucleus, with more than 90% of the cells presenting with nuclear DLC1 localization after 2 hours of LMB treatment (Fig. 7D). Finally, we examined whether 14-3-3γ coexpression was capable of preventing DLC1 nucleocytoplasmic shuttling. Cells that expressed only DLC1 demonstrated LMB-induced nuclear accumulation as expected, whereas DLC1 was

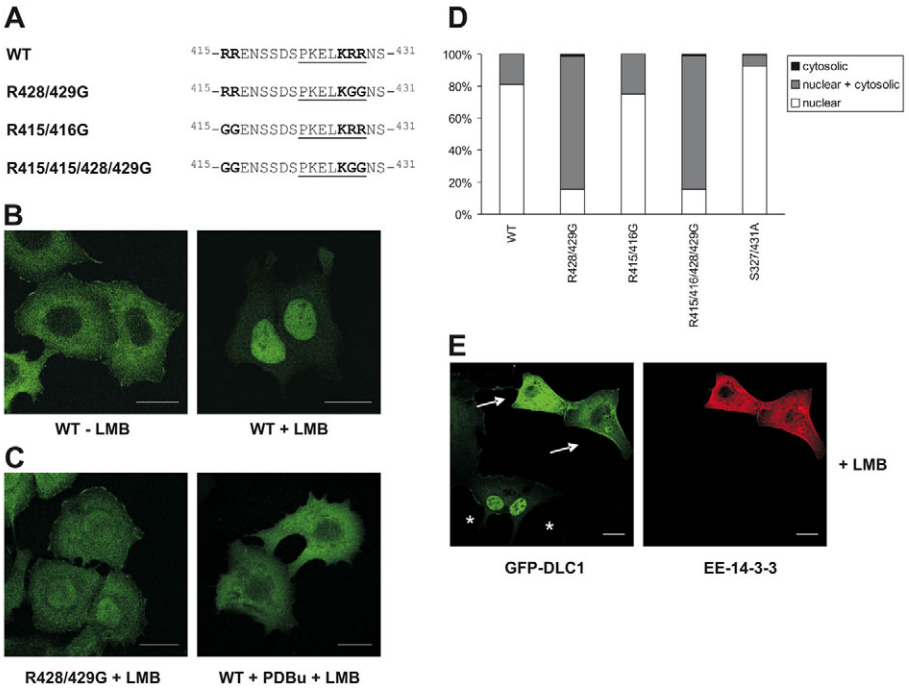

Fig. 7. Binding of 14-3-3 proteins inhibits DLC1 nuclear transport mediated by an NLS spanning residues 423-429. (A) Schematic representation of DLC1 WT and Arg-Gly exchange mutants. The putative bipartite NLS is highlighted in bold, the pat7 NLS is underlined. (B,C) MCF7 cells were transiently transfected with GFP-tagged DLC1 WT or R428/429G and treated with LMB for 2 hours prior to fixation. (C) Cells were stimulated with 100 nM PDBu prior to LMB addition. (D) MCF7 cells transiently expressing the indicated GFP-tagged DLC1 variants were treated with LMB for 2 hours and fixed. The number of cells displaying mainly cytosolic, homogenous (nuclear + cytosolic) or mainly nuclear protein distribution was determined by counting 100 cells each in random microscopic fields. Values correspond to the mean of two independent experiments. (E) COS7 cells were transiently transfected with both GFP-tagged DLC1 and EE-14-3-3γ expression vectors and treated with 10 ng/ml LMB for 1 hour prior to fixation and staining with EE-specific antibody. Cells expressing only DLC1 are indicated with asterisks, cells expressing DLC1 and 14-3-3γ are marked with arrows. All images are single confocal sections. Scale bars: 20 μm.

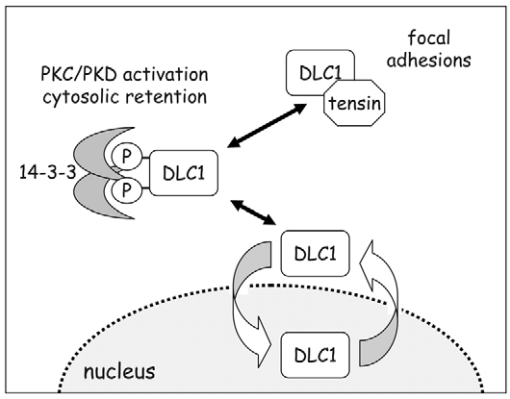

Fig. 8. Regulation of DLC1 by 14-3-3 proteins. Activation of PKC/PKD kinases leads to DLC1 phosphorylation on sites that include Ser327 and Ser431. This creates binding sites for 14-3-3 adaptor proteins, whereby DLC1 RhoGAP activity is inhibited, most likely by cytosolic sequestration. 14-3-3 binding furthermore masks a nuclear localization signal spanning residues 423-429, thus preventing DLC1 nucleocytoplasmic shuttling.

retained in the cytoplasm in cells expressing both proteins (Fig. 7E). Phorbol-ester-induced activation of PKC and PKD kinases thus leads to DLC1 phosphorylation on several sites, which include Ser327 and Ser431. This promotes 14-3-3-adaptor-protein binding, whereby DLC1 RhoGAP activity is inhibited and the protein sequestered in the cytoplasm by masking of a novel NLS (see Fig. 8 for a model).

Discussion

Here we report a novel phosphorylation-dependent interaction between the RhoGAP protein DLC1 and 14-3-3 adaptor proteins. In GST pull-down assays, DLC1 interacted with all 14-3-3 isoforms except 14-3-3σ. The latter isoform is unique in that it is primarily expressed in epithelial tissues and appears to be involved in cellular responses to DNA damage and in human oncogenesis. Structural analysis revealed that 14-3-3σ preferentially homodimerizes, and that residues specific to this isoform determine substrate selection (Wilker et al., 2005). The other 14-3-3 members are ubiquitously expressed, readily form homo- and heterodimers, and could therefore potentially participate in the regulation of DLC1 function. Recent global 14-3-3 interaction screens identified many proteins involved in cytoskeletal regulation, including several GEFs and GAPs, suggesting a general role for 14-3-3 proteins in cellular architecture (Angrand et al., 2006; Jin et al., 2004; Rubio et al., 2004). The RhoGEF AKAP-Lbc, for example, is phosphorylated by PKA, which recruits 14-3-3 proteins and suppresses its GEF activity (Diviani et al., 2004; Jin et al., 2004). DLC1 was not isolated in any of these screens, possibly because of low expression levels and/or low basal phosphorylation.

Inhibition of cellular protein phosphatase activity with okadaic acid stabilized DLC1 in a hyperphosphorylated form, as deduced from the dramatic shift in electrophoretic mobility, which suggests phosphorylation on several sites. We provide evidence that phorbol ester stimulation promotes DLC1 phosphorylation on Ser327 and Ser431 and at least one additional site to generate cooperating 14-3-3 binding motifs. Mutation of both Ser327 and Ser431 in DLC1 was necessary to impair binding of 14-3-3 proteins. Owing to the dimeric nature of 14-3-3 proteins, protein contact through multiple sites is a common theme. In fact, a phosphopeptide with two binding motifs binds 14-3-3 proteins with a 30-fold greater affinity than a phosphopeptide containing a single motif (Yaffe et al., 1997). DLC1 association with 14-3-3 proteins was blocked by pharmacological inhibition of the phorbol-ester-responsive kinases PKC and PKD. Based on our in vitro kinase assay results, Ser327 and Ser431 are probably phosphorylated by PKD directly. The additional phorbol-ester-induced 14-3-3 binding site that remains to be identified appears to be phosphorylated independently of PKD. It is possible that this site is phosphorylated by PKC but it could also be a substrate of a kinase activated indirectly by phorbol ester stimulation. Ser322 in rat DLC1 is phosphorylated in response to insulin (Hers et al., 2006) and according to Scansite could generate a 14-3-3 binding site. However, insulin treatment of HEK293T cells expressing human DLC1 did not enhance 14-3-3 binding as judged by GST pull-downs. Moreover, alanine mutation of the equivalent serine in the human protein had no influence on DLC1 interaction with 14-3-3 proteins (data not shown), making it unlikely that this site is involved in 14-3-3 binding.

14-3-3 proteins often regulate cellular processes by modulating target protein localization. Here we provide evidence for a pat7 NLS spanning residues 423-429 that appears to be masked by phorbol-ester-induced DLC1 phosphorylation and 14-3-3 binding. Inactivation of this NLS by exchange of critical arginine residues impaired but did not prevent nuclear import. This suggests that at least one additional NLS exists that contributes to DLC1 nuclear shuttling. The function of DLC1 in the nucleus remains to be defined. The C-terminal polybasic region of Rac1 was shown to function as a NLS. In RhoA, this region does not have functional NLS activity, but other Rho GTPases that are transported to the nucleus via their polybasic region may serve as substrates for DLC1 (Williams, 2003). Alternatively, DLC1 may have non-GTPase substrates in this compartment. Rat DLC1 was originally reported to bind and activate phospholipase C δ1 (PLC-δ1) (Homma and Emori, 1995), an enzyme that translocates to the nucleus during specific phases of the cell cycle (Stallings et al., 2005). However, in overexpression studies, human DLC1 did not stimulate phospholipid hydrolysis activity of PLC-δ1 (Healy et al., 2008). The nucleus may alternatively restrict the amount of DLC1 available in the cytoplasm to control cellular Rho-GTP levels. In a recent publication, nuclear translocation of DLC1 was proposed to be associated with apoptosis by a yet unknown mechanism (Yuan et al., 2007). The authors further claimed that nuclear entry was mediated by a bipartite NLS involving Arg415 and Arg416. This contrasts with our data, which do not support any contribution of these residues to nuclear transport. The different results could be explained by the substitution of Arg415-Arg416 with bulky tryptophan residues (as opposed to glycines used in our study), which might alter conformation and functionality of the pat7 NLS located further downstream.

It has now become apparent that 14-3-3-mediated compartmentalization is not restricted to nuclear proteins but is a regulatory mechanism that also applies to proteins that shuttle from the cytoplasm to the plasma membrane. Ras is a membrane-bound small GTPase, with many of its effectors and regulators being cytosolic. To exert their function, these molecules must translocate to the cell surface. Interestingly, PKD has been implicated in the regulation of such a translocation process by direct phosphorylation of the Ras effector RIN1 on Ser351, which creates a 14-3-3 binding site (Wang et al., 2002). When 14-3-3 binding is disrupted, RIN1 exhibits increased association with the plasma membrane and competes with Raf for Ras binding. We observed that DLC1

expressed in MCF7 cells was associated with focal adhesions and the plasma membrane, in line with previous reports. Coexpression of 14-3-3 proteins had no obvious influence on DLC1 targeting to these subcellular compartments, as judged by indirect immunofluorescence and cell fractionations (data not shown). However, because 14-3-3 binding requires DLC1 phosphorylation, a low stoichiometry of phosphorylation might impede visualization of location changes. A DLC1 variant that constitutively binds 14-3-3 proteins would be required to address this issue, but the attempt to generate such a mutant by introducing negatively charged amino acids was not successful. In cells treated with phorbol ester, DLC1 was mainly cytosolic, suggesting exclusion of the phosphorylated protein from focal adhesions. However, phorbol ester treatment appeared to generally affect cellular cytoskeletal architecture as judged by immunostaining of the focal adhesion protein paxillin (supplementary material Fig. S1). 14-3-3γ and τ expressed in MCF7 cells colocalized with DLC1 in the cytosol and at the plasma membrane, but were excluded from focal adhesions, suggesting that interaction with 14-3-3 proteins occurs with the soluble and membrane-bound DLC1 pool. If association with 14-3-3 proteins does not relocate DLC1 from the plasma membrane, it is also possible that inhibition of DLC1 function is due to a conformational change elicited by 14-3-3 binding that blocks its GAP activity.

DLC1 is directed to focal adhesions by interacting with tensin proteins and this localization has been proposed to be associated with biological activity (Liao et al., 2007; Qian et al., 2007; Yam et al., 2006). Although still active with respect to GAP function, DLC1 mutants deficient in tensin binding were no longer able to suppress cell growth. The four tensin members have a phosphotyrosine-binding domain that mediates interaction with the cytoplasmic tails of β-integrins (Lo, 2004). Tensin-1, tensin-2 and tensin-3 (TENS1-TENS3) also interact with actin at multiple sites in their N-terminal region, as opposed to the shorter tensin-4 (also known as cten and TENS4), which lacks the actin-binding sites. Interaction with tensins was mapped to residues 440-445 in DLC1 and occurs via the tensin Src homology 2 (SH2) domain (Liao et al., 2007; Qian et al., 2007). This tensin-binding sequence in DLC1 is adjacent to the 14-3-3 binding site harboring Ser431, prompting the idea that 14-3-3 proteins might compete with tensin interaction. However, phorbol-ester-induced phosphorylation of DLC1 did not suppress interaction with the tensin-4 SH2 domain in GST pull-down assays (data not shown). Since the tensin phosphotyrosine-binding domain has also been implicated in interacting with the DLC1 N-terminus (Qian et al., 2007; Yam et al., 2006), it remains to be tested with full-length proteins whether 14-3-3 and tensin binding are mutually exclusive.

Formation of complexes with 14-3-3 proteins, at least downstream of phorbol-ester-induced intracellular signaling, appears to be a regulatory mechanism restricted to DLC1. GST pull-down assays did not reveal any PDBu-induced 14-3-3 binding to the structurally related DLC2 or DLC3 proteins. This agrees with the fact that Ser327 and Ser431 are not conserved in DLC2 and DLC3, nor is the NLS identified in this study, suggesting distinct modes of regulation provided by the non-conserved linker regions, despite their overlapping substrate specificities in vitro. PKD participates in the regulation of diverse cellular processes, including cell migration, proliferation and secretory transport from the *trans*-Golgi compartment to the plasma membrane. To accomplish these diverse functions, depending on the cell type and external stimulus, PKD localizes to the Golgi complex, the plasma membrane, and is also found in the nucleus (Rykx et al., 2003; Wang, 2006). In future studies, it will be of particular interest to define the compartment in which PKD phosphorylates DLC1 and to extend our investigations on physiological conditions that trigger inhibitory DLC1 phosphorylation and association with 14-3-3 proteins, with a special focus on cell adhesion processes, to maintain local Rho signaling.

Materials and Methods

Antibodies and reagents

Antibodies used were: mouse anti-Flag mAb (Sigma), mouse anti-GFP mAb (Roche), goat anti-GST pAb (GE Healthcare), mouse anti-GluGlu mAb (Hiss Diagnostics), rabbit anti-14-3-3 pAb (K19), mouse anti-14-3-3 mAb (H8), rabbit anti-GFP pAb (FL) and mouse anti-Rho mAb (26C4) (Santa Cruz Biotechnology), mouse anti-DLC1 mAb (BD), mouse anti-α-tubulin mAb (Sigma). Mouse anti-Myc mAb, clone 9E10, and mouse anti-HA mAb, clone 12CA5, were kindly provided by Heiner Böttinger (University of Stuttgart, Germany). HRP-labeled secondary anti-mouse and anti-rabbit IgG antibodies were from Amersham, HRP-labeled secondary anti-goat IgG antibody was from Santa Cruz Biotechnology; alkaline-phosphatase-labeled secondary anti-goat IgG antibody was from Sigma; Alexa Fluor 546-labeled secondary anti-mouse IgG antibody was from Molecular Probes. Staurosporine and okadaic acid were from Alexis; Gö6983, Gö6976, PDBu and doxycycline were from Calbiochem, and LMB was from Biomol.

DNA constructs

pCS2+MT-DLC1 encoding Myc-tagged DLC1 was kindly provided by Irene Ng (The University of Hong Kong, China). Full-length DLC1 cDNA was amplified by PCR using pCS2+MT-DLC1 as a template with primers containing *Bam*HI restriction sites (DLC1-for, 5′-cgcggatcc tgcagaaagaagccggaccc-3′ and DLC1-rev, 5′-cgcggatcctcacc-tagatttggtgtctttgg-3′) and cloned into Flag-pEFrPGKpuro, pEGFPC1, and pcDNA5/FRT/TO-GFP (see below) vectors. Truncated DLC1 variants were generated by PCR amplification using the following primers: DLC1-ΔSAM (5′-cgcgg-atccattagtcctcatcggaaacgaag-3′ and DLC1-rev)**;** DLC1-ΔStart (DLC1-for and 5′-cgc-ggatcctcacaggtgcccgagtgcttc-3′); DLC1-ΔC (DLC1-for and 5′-cgcggatcctcacatgaactt-gggcacggcc-3′); DLC-ΔN (5′-cgcggatccaagaggatcaaggttccagac-3′ and DLC1-rev). DLC1 point mutants were generated by QuikChange site-directed PCR mutagenesis according to the manufacturer's instructions (Stratagene). The forward primers used were: S236A-for (5′-gctgaaacggatggaggccctgaagctcaagagc-3′); S327A-for (5′-gttacg-aggacccgggccctcagtgcgtgc-3′); S419A-for (5′-cctcaggagggaaaacgctagcgacagcccc-aagg-3′); S431A-for (5′-ctgaagagacgcaatgcttccagctccatgagc-3′); R415/416G-for (5′-gccacatcagcctcgggggggaaaacagtagcg-3′); R428/429G-for (5′-cccaaggaactgaagggagg-caattcttccagctcc-3′). To generate the inducible *DLC1* expression vector pcDNA5/FRT/TO-GFP-DLC1, the enhanced GFP cDNA was excised from the pEGFPC1 vector with *Eco*47III and *Ksp*A1 and ligated with pcDNA5/FRT/TO digested with *Mss*I. The *DLC1* cDNA was then inserted in frame as a *Bam*HI fragment. All amplified cDNAs were verified by sequencing. Oligonucleotides were purchased from MWG Biotech. pEGFPN1-PKD1, pGEX-14-3-3τ WT and R56/60, HA-tagged 14-3-3τ and EE-tagged 14-3-3γ and ζ in pEF vectors have been described previously (Hausser et al., 2005; Hausser et al., 2006; Olayioye et al., 2003). pGEX-DLC1(aa242-569)-WT, pGEX-DLC1-S327/431A and pGEX-DLC1-S327/419/431A constructs were generated by subcloning of *Ecl*136II fragments from the respective full-length constructs into the pGEX6P1 vector linearized with *Sma*I.

Cell culture and transfection

HEK293T, COS7, MCF7 and MDAMB231 cells were grown in RPMI containing 10% FCS in a humidified atmosphere containing 5% CO_2. HEK293T cells were transfected using TransIT293 reagent (Mirus). For immunofluorescence, MCF7 and COS7 cells were grown on glass coverslips and transfected with Lipofectamine 2000 (Invitrogen). Flp-In T-Rex HEK293 cells (Invitrogen) were grown in DMEM containing 10% FCS, 100 μg/ml zeocin and 15 μg/ml blasticidin. These cells stably express the Tet repressor and contain a single Flp Recombination Target (FRT) site and were used to generate the Flp-In-DLC1 line. Cells were cotransfected with pcDNA5/FRT/TO-GFP-DLC1 and the Flp recombinase expression plasmid pOG44 at a ratio of 1:10 and then selected with 100 μg/ml hygromycin. Stable MCF7 lines were generated by transfection of vectors encoding Flag-tagged DLC1 wild type and S327/431, or empty vector as a control, followed by selection with 1.5 μg/ml puromycin for 10 days.

Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde for 10 minutes, washed and incubated with PBS containing 0.1 M glycine for 15 minutes. Cells were permeabilized with PBS containing 0.1% Triton for 5 minutes and blocked with 5% goat serum in PBS containing 0.1% Tween-20 for 30 minutes. Cells were then incubated with primary antibody diluted in blocking buffer for 2 hours, followed by incubation with secondary antibody diluted in blocking buffer for 1 hour. Coverslips were mounted in Fluoromount G (Southern Biotechnology) and analyzed on a confocal laser-

scanning microscope (TCS SL, Leica) using 488 nm and 543 nm excitation and a 40.0/1.25 HCX PL APO objective lens.

Bacterial expression of GST proteins

E. coli were transformed with pGEX vectors encoding GST-14-3-3 proteins, DLC1(aa 242-569) variants or GST alone and expression was induced with 0.1 mM IPTG for 4 hours at 37°C. The bacterial cultures were harvested and pellets were resuspended in PBS containing Complete protease inhibitors (Roche). The suspension was then sonicated 3× for 10 seconds on ice, Triton X-100 was added to a final concentration of 1% and the lysate centrifuged for 10 minutes at 8000 ***g***. Purification of GST-tagged proteins was performed with glutathione resin (GE Healthcare). The resin was washed with PBS and the purity and amount of bound GST proteins was then determined by SDS-PAGE and Coomassie blue staining.

Pull-downs, immunoprecipitation and western blotting

Whole-cell extracts were obtained by solubilizing cells in Triton X-100 extraction buffer (TEB) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete protease inhibitors). Lysates were clarified by centrifugation at 16,000 ***g*** for 10 minutes. Pull-downs were performed by incubating whole-cell extracts with immobilized GST proteins for 2 hours. Beads were washed three times with TEB. For immunoprecipitations, equal amounts of protein were incubated with specific antibodies for 2 hours on ice. Immune complexes were collected with protein-G-Sepharose (GE Healthcare) and washed three times with TEB. In the case of stably expressed Flag-DLC1, precipitation was overnight and washes were with TEB containing 0.5% Triton X-100. Precipitated proteins were released by boiling in sample buffer, subjected to SDS-PAGE and blotted onto PVDF membranes (Roth). After blocking with 0.5% blocking reagent (Roche) in PBS containing 0.1% Tween 20, filters were probed with specific antibodies. Proteins were visualized with HRP-coupled secondary antibody using the ECL detection system (Pierce) or alkaline phosphatase-coupled secondary antibody and NBT/BCIP as a substrate.

Kinase assays

Equal amounts of the purified GST-DLC1(aa242-569) proteins were mixed with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT) containing 2 μCi [γ-^{32}P]ATP and incubated for 5 minutes at 37°C in the presence or absence of 50 ng purified Myc-PKD1 (Dieterich et al., 1996). Samples were then resolved by SDS-PAGE, transferred to membrane and analyzed on a PhosphoImager (Molecular Dynamics), followed by immunoblotting.

Luciferase reporter assays

HEK293 Flp-In-DLC1 cells were grown on collagen-coated 24-well dishes and transfected with 50 ng each of the 3DA.Luc firefly luciferase reporter containing three SRF binding elements, pRL-TK, a *Renilla* luciferase plasmid under the control of the thymidine kinase promoter, and pEF-HA-14-3-3τ. After serum starvation overnight, DLC1 expression was switched on by addition of 10 ng/ml doxycycline and, 4 hours later, cells were stimulated with 100 nM PDBu for 4 hours. Cells were lysed with 300 μl passive lysis buffer (Promega) and luciferase activities in 10 μl lysate were measured by addition of 50 μl firefly substrate (470 μM D-luciferin, 530 μM ATP, 270 μM coenzyme A, 33 mM DTT, 20 mM Tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, pH 7.8), followed by addition of 100 μl *Renilla* substrate (0.7 μM coelenterazine, 2.2 mM Na_2EDTA, 0.44 mg/ml bovine serum albumin, 1.1 M NaCl, 1.3 mM NaN_3, 0.22 M potassium phosphate buffer, pH 5.0). Luminescence was measured with a Tecan Infinite 200M plate reader.

Rho activity measurements

HEK293T cells transiently expressing the Raichu-RhoA biosensor were lysed in 50 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 5 mM sodium fluoride and 0.5% Triton X-100 and debris was removed by centrifugation at 16,000 ***g*** for 10 minutes. Emission ratios (FRET/CFP) were determined by measuring CFP and YFP fluorescence after background subtraction at 475 and 530 nm, respectively, using a Tecan Infinite 200M plate reader (excitation, 433 nm).

MTT assays

Approximately 2000 cells in 150 μl medium were plated into 96-well plates (n=5) and incubated with 15 μl of 3-(4,5-dimethylthiazol-2yl-)2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml) for 2 hours. Cells were lysed in 100 μl 50% dimethylformamide containing 10% SDS and absorbance at 595 nm was determined with background subtraction at 655 nm and absorbance of medium alone using a SpectraMax 340PC^{384} reader (Molecular Devices).

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