Molecular Biology of the Cell
Vol. 20, 4010–4020, September 15, 2009

Par-4 Is an Essential Downstream Target of DAP-like Kinase (Dlk) in Dlk/Par-4–mediated Apoptosis

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Submitted March 2, 2009; Revised June 23, 2009; Accepted July 14, 2009
Monitoring Editor: Kunxin Luo

Prostate apoptosis response-4 (Par-4) was initially identified as a gene product up-regulated in prostate cancer cells undergoing apoptosis. In rat fibroblasts, coexpression of Par-4 and its interaction partner DAP-like kinase (Dlk, which is also known as zipper-interacting protein kinase [ZIPK]) induces relocation of the kinase from the nucleus to the actin filament system, followed by extensive myosin light chain (MLC) phosphorylation and induction of apoptosis. Our analyses show that the synergistic proapoptotic effect of Dlk/Par-4 complexes is abrogated when either Dlk/Par-4 interaction or Dlk kinase activity is impaired. In vitro phosphorylation assays employing Dlk and Par-4 phosphorylation mutants carrying alanine substitutions for residues S154, T155, S220, or S249, respectively, identified T155 as the major Par-4 phosphorylation site of Dlk. Coexpression experiments in REF52.2 cells revealed that phosphorylation of Par-4 at T155 by Dlk was essential for apoptosis induction in vivo. In the presence of the Par-4 T155A mutant Dlk was partially recruited to actin filaments but resided mainly in the nucleus. Consequently, apoptosis was not induced in Dlk/Par-4 T155A–expressing cells. In vivo phosphorylation of Par-4 at T155 was demonstrated with a phospho-specific Par-4 antibody. Our results demonstrate that Dlk-mediated phosphorylation of Par-4 at T155 is a crucial event in Dlk/Par-4-induced apoptosis.

INTRODUCTION

Apoptosis, or programmed cell death, is a tightly regulated process that plays a crucial role in development and tissue homeostasis (Vaux and Strasser, 1996; Le Bras *et al.*, 2005). In addition, apoptosis is an important defense mechanism that protects organisms from pathogenesis of a variety of diseases including cancer, autoimmune disease, neurodegenerative disorders, and viral infections (Igney and Krammer, 2002; Debatin and Krammer, 2004; Clarke *et al.*, 2005). The signaling pathways that lead to the initiation of programmed cell death converge in the activation of a proteolytic cascade involving aspartate-specific caspases that mediate the coordinated elimination of the apoptotic cell (Garrido and Kroemer, 2004; Riedl and Shi, 2004). Proapoptotic kinases play an essential role in the apoptotic signaling pathway. For example, overexpression of ASK1 (apoptosis signal-regulating kinase1) or the constitutively active form of ASK1 results in apoptosis induction in various cell types (Ichijo *et al.*, 1997; Chang *et al.*, 1998; Saitoh *et al.*, 1998). Furthermore, it has been reported that a kinase-inactive mutant of ASK1 inhibits apoptosis induced by tumor necrosis factor-α, Fas ligation, or anticancer drugs (Chen *et al.*, 1999; Kanamoto *et al.*, 2000). Other kinases that can modulate the apoptotic response are RIP (receptor interacting protein), PKCδ (protein kinase C delta), PITSLRE (member of the cyclin-dependent kinase superfamily), and DAPK (death-associated protein kinase; Deiss *et al.*, 1995; Emoto *et al.*, 1995; Lahti *et al.*, 1995; Stanger *et al.*, 1995; Meylan and Tschopp, 2005).

The DAPK family is a recently discovered group of highly related serine/threonine kinases that are involved in cell death signaling (Cohen and Kimchi, 2001; Kimchi, 2001). Three protein kinases of the DAPK family share 80% amino acid identity within their catalytic domains: DAPK, DAPK-related protein 1 (DRP-1), and DAP-like kinase (Dlk), which is also termed zipper-interacting protein kinase (ZIPK) or DAPK3 (Kawai *et al.*, 1998; Kögel *et al.*, 1998; Inbal *et al.*, 2000; Shoval *et al.*, 2007). A common feature in cell death induced by DAPK family members is the process of membrane blebbing which has been attributed to increased phosphorylation of the regulatory light chain of myosin II (MLC). MLC has been shown to be an in vitro substrate for all DAPK family members (Cohen *et al.*, 1997; Kawai *et al.*, 1998; Kögel *et al.*, 1998; Sanjo *et al.*, 1998; Kawai *et al.*, 1999; Inbal *et al.*, 2000; Debatin and Krammer, 2004). Experimental evidence further suggests that Dlk can mediate MLC phosphorylation in rat fibroblasts (Vetterkind *et al.*, 2005b). This kinase has been implicated in apoptotic (Kögel *et al.*, 1999; Page *et al.*, 1999a; Kawai *et al.*, 2003; Shani *et al.*, 2004) as well as mitotic processes (Engemann *et al.*, 2002; Preuss *et al.*, 2003a,b).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–02–0173) on July 22, 2009.

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Abbreviations used: Par-4, prostate apoptosis response-4; Dlk, DAP-like kinase; MLC, myosin light chain.

Although mitotic functions require a nuclear localization of the kinase, the proapoptotic potential of Dlk appears to correlate with enhanced cytoplasmic localization (Kögel *et al.*, 1999; Page *et al.*, 1999a; Shani *et al.*, 2004). For example, a C-terminal Dlk deletion mutant defective for nuclear transport exhibited pronounced colocalization with actin filaments and high apoptotic activity in rat fibroblasts (Kögel *et al.*, 1999). Furthermore, it has been shown that DAPK-mediated phosphorylation of ZIPK (the human orthologue of Dlk) results in predominantly cytoplasmic localization and greater cell death–inducing potency (Shani *et al.*, 2004). The translocation of nuclear Dlk to the cytoplasm implies that Dlk-mediated apoptosis might depend on phosphorylation of a cytoplasmic downstream target.

A good candidate in this context is the proapoptotic protein Par-4 (prostate-apoptosis response-4) that was originally identified as an early response gene induced during apoptosis in prostate cancer cells (Sells *et al.*, 1994). Protein complexes containing both Dlk and Par-4 have successfully been isolated from various cell types derived from different species (Page *et al.*, 1999a; Kawai *et al.*, 2003; Vetterkind and Morgan, 2009). However, direct interaction between the two proteins has only unambiguously been demonstrated for the murine system so far, where Par-4 is a direct interaction partner and in vitro substrate of Dlk (Page *et al.*, 1999a). In fact, a recent report (Shoval *et al.*, 2007) suggests that Par-4 binding may be a special feature of murine Dlk necessary to compensate for the evolutionary loss of regulatory phosphorylation sites, the phosphorylation of which is essential for cytoplasmic localization of ZIPK, the human orthologue of Dlk (Graves *et al.*, 2005). Consistent with this hypothesis, coexpression of Par-4 and murine Dlk in rat fibroblasts leads to cytoplasmic accumulation of the kinase and Par-4–mediated recruitment to the actin cytoskeleton. Furthermore, enhanced MLC phosphorylation associated with a dramatic reorganization of the actin filament system and induction of apoptosis was observed (Vetterkind *et al.*, 2005b). At present it is not entirely clear whether Dlk/Par-4 complex formation results from cytoplasmic retention of Dlk before nuclear entry or involves export of nuclear Dlk/Par-4 complexes. Both scenarios are conceivable because it has been shown that Par-4 localizes to the cytoplasm and the nucleus, albeit to different extents, depending on cell type and tissue (Boghaert *et al.*, 1997; Sells *et al.*, 1997; Guo *et al.*, 1998; Page *et al.*, 1999a; El-Guendy *et al.*, 2003). As a consequence, it has been reported that Par-4 interacts with nuclear as well as cytoplasmic proteins (Diaz-Meco *et al.*, 1996; Johnstone *et al.*, 1996; Page *et al.*, 1999a; Roussigne *et al.*, 2003). In a nuclear complex with the transcription factor Wilms' tumor protein 1 (WT1), Par-4 acts as a transcriptional coregulator that represses the expression of the *bcl-2* gene (Cheema *et al.*, 2003). On the other hand, one of the cytoplasmic functions described for Par-4 is the inhibition of the protein kinase C isoform ζ (PKCζ), thereby repressing PKCζ-mediated prosurvival signaling (Diaz-Meco *et al.*, 1996; Wang *et al.*, 2005).

The apoptotic activity of Par-4 has been confined to the C-terminal part of the protein, which contains a leucine zipper domain partially overlapping a death domain (Diaz-Meco *et al.*, 1996; Johnstone *et al.*, 1996; Rangnekar, 1998). Deletion of the leucine zipper resulted in the loss of proapoptotic activity and, conversely, overexpression of the leucine zipper domain had a dominant-negative effect by abrogating the proapoptotic function of full-length Par-4 (Sells *et al.*, 1997). Ectopic expression of Par-4 is sufficient to induce apoptosis in several types of tumor cells (Chakraborty *et al.*, 2001; Lucas *et al.*, 2001; El-Guendy and Rangnekar, 2003), and it has been shown that a central domain of Par-4 that included NLS2 but lacked the leucine zipper domain (amino acids 137–195) can induce apoptosis in cancer cells (El-Guendy *et al.*, 2003). This domain called SAC (for selective for apoptosis induction in cancer cells) contains a threonine residue (T155) that can be phosphorylated by protein kinase A (PKA) in vitro and appears to be critical for the proapoptotic function of Par-4 in cancer cells (Gurumurthy *et al.*, 2005). In contrast, phosphorylation of a serine residue (S249) outside SAC near the Par-4 death domain by the kinase Akt (or protein kinase B) promotes cancer cell survival through selective sequestration of S249 phosphorylated Par-4 by binding to 14-3-3 proteins (Goswami *et al.*, 2005). Taken together, these investigations suggest that phosphorylation is a key regulatory event which modulates Par-4 function.

Even though the cooperative effect of Dlk and Par-4 has been well documented in the past, the molecular mechanism how Dlk/Par-4 complexes trigger apoptosis in rat fibroblasts is not fully understood. In the present study we analyzed the functional relationship of Par-4 and Dlk in REF52.2 cells investigating Par-4 phosphorylation by Dlk and its cellular effects.

MATERIALS AND METHODS

Cell Culture and Transfection

Rat embryo fibroblasts (line REF52.2) were maintained in DMEM (Invitrogen, Karlsruhe, Germany) with 10% FCS (PAA Laboratories, Vienna, Austria), 100 U/ml penicillin, and 100 μg/ml streptomycin. For transfection experiments, cells were seeded at 6×10^5/100-mm culture dish or at 1.7×10^4/coverslip and transiently transfected with 300 ng of expression vectors using the jetPEI transfection reagent (PolyPlus, Illkirch, France) according to the manufacturer's protocol.

Construction of Plasmids

Cloning of Par-4-GFP, Par-4-CFP, FLAG-Par-4, Par-4 L3-GFP (green fluorescent protein), GFP-Dlk, and GFP-Dlk K42A has been described previously (Kögel *et al.*, 1998; Kögel *et al.*, 1999; Boosen *et al.*, 2005; Vetterkind *et al.*, 2005a,b). The cDNA sequence of Dlk was excised from the pGEX vector (Kögel *et al.*, 1998) with restriction enzymes BamHI and XhoI and inserted into the vector pCMV-Tag2B (Stratagene, La Jolla, CA) to construct the FLAG-Dlk expression vector. For the generation of the yellow fluorescent protein (YFP)-Dlk construct, the cDNA sequence of enhanced YFP (EYFP) was excised from the pEYFP-C1 vector (BD Biosciences Clontech, Heidelberg, Germany) with restriction enzymes BsrGI and AgeI and subsequently subcloned into the GFP-Dlk vector. The Par-4 phosphorylation mutants carrying either an exchange of threonine or serine to alanine or both exchanges were generated by PCR site-directed mutagenesis using the Par-4 wild-type (wt) cDNA as template. In the first reaction the oligonucleotides Par-4 103–123 5′-AGCACCACGGACTTCCTGGAG-3′ forward and Par-4 AgeI 5′-CCACCGGTCCCCTTGTCAGCTGCCC-3′ reverse were used with primers harboring the desired mutation generating overlapping PCR fragments: Par-4 S154: forward, 5′-AAGCGCCGCGCTACCGGCGTG-3′ and reverse, 5′-CACGCCGGTAGCGCGGCGCTT-3′; Par-4 T155A: forward, 5′-AAGCGCCGCTCCGCTGGCGTGGTCAAC-3′ and reverse, 5′-GTTGACCACGCCAGCGGAGCG-3′; Par-4 S154A/T155A: forward, 5′- AAGCGCCGCGCAGCTGGCGTGGTCAAC-3′ and reverse, 5′-CACGCCAGCTGCGCGGCG-3′; Par-4 S220A: forward, 5′-GCAGATACAAAGCCACAATCAGTGC-3′ and reverse, 5′-GCACTGATTGTGGCTTTGTATCTGC-3′; Par-4 T155D: forward 5′-AAGCGCCGCTCCGACGGCGTGGTCAAC-3′ and reverse, 5′-GTTGACCACGCCGTCGGAGCG-3′; and Par-4 T155E: forward, 5′-AAGCGCCGCTCCGAGGGCGTGGTCAAC-3′ and reverse, 5′-GTTGACCACGCCCTCGGAGCG-3′.

In the second PCR reaction the resulting DNA fragments were assembled by PCR using the oligonucleotides Par-4 103–123 5′-AGCACCACGGACTTCCTGGAG-3′ forward and Par-4 AgeI 5′-CCACCGGTCCCCTTGTCAGCTGCCC-3′ reverse. The generated PCR products were digested with PshAI and EcoRV (for S154A and T155A), with PshAI and AccI (for S154/T155A) or with BssHII and AccI (for S220A) and subcloned into the Par-4 wt-GFP expression vector digested with restriction enzymes accordingly. For the generation of Par-4 mutant S249A the PCR reaction was carried out with oligonucleotides Par-4 S249A 5′-GCTTCAGTAGACACAACAGAGATACCGCCGCG-3′ forward and Par-4 AgeI 5′-CCACCGGTCCCCTTGTCAGCTGCCC-3′ reverse. The amplified PCR product was digested with AccI and AgeI and subsequently subcloned into the Par-4 wt-GFP expression vector. All mutations were confirmed by subsequent sequencing (AGOWA, Berlin, Germany).

Protein Expression and Purification

Cloning of the strep-tagged recombinant Par-4 wt protein has been described previously (Vetterkind *et al.*, 2005b). For the generation of the strep-tagged Par-4 phosphorylation mutants S154A, T155A, S154/T155A, S220A, and S249A, the cDNA sequence was excised from the Par-4-GFP vector, respectively, with restriction enzymes BssHIIand EcoRV and subsequently subcloned into the pET23a(+) Par-4 wt vector. Strep-tagged full-length Par-4 and Par-4 mutants were expressed in the *Escherichia coli* strain BL21-CodonPlus DE3 (Stratagene). Bacteria were transformed with either Par-4 expression vector and grown in DYT-medium at 37°C. Protein expression was induced in late log phase with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Bacteria were harvested 3 h after induction and solubilized in buffer (50 mM Tris, pH 7.0, and 200 mM $MgCl_2$) by ultrasonic disruption. Lysates were cleared from cell debris by centrifugation. Recombinant proteins were purified from the cell lysates using StrepTactin Sepharose (IBA, Göttingen, Germany) essentially according to the manufacturer's instructions.

In Vitro Phosphorylation Assay and Phosphoamino Acid Analysis

In vitro phosphorylation assays were carried out with purified recombinant Par-4 and Dlk in the presence of [γ-^{32}P]ATP essentially as described by Page *et al.* (1999a). For the phosphorylation reactions of Par-4 with PKA, 0.5 μM catalytic subunit of PKA (Calbiochem, La Jolla, CA) was used in the assay. In vitro–phosphorylated, radiolabeled Par-4 was separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane. Radioactive bands were identified by autoradiography, isolated, and subjected to acid hydrolysis according to Preuss *et al.* (2003b). Phospho-serine and phospho-threonine (Sigma, St. Louis, MO) were used as internal standards and stained with ninhydrin. Radiolabeled phosphoamino acids were detected by autoradiography.

Antibody Generation

A phospho-specific antibody was raised in rabbits against a synthetic phospho-peptide of the sequence KRRSpTGVVN corresponding to residues 151–159 of rat Par-4 (Pineda Antibody Service, Berlin, Germany). The polyclonal anti-Phospho-Par-4(T155) antibody [denoted Par-4(P)T155] was affinity-purified on a peptide column.

Fluorescence Microscopy

For immunofluorescence analysis, cells were fixed 24 h after transfection with 3% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were then treated with 5% nonfat dry milk for 1 h and stained with the Par-4(P)T155 antibody (Pineda Antibody Service) at 1:2000–1:8000 dilution and the mouse monoclonal anti-FLAG M2 antibody (Stratagene) at 1:5000 dilution for 1 h at room temperature. As secondary antibodies, Cy3-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dianova, Hamburg, Germany) were used at 1:2000 dilution and incubated for 30 min. Actin filaments were stained with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (Sigma) at room temperature for 15 min. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) for 15 min and subsequent washed with PBS. Cells were examined with an Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CCD camera using filters optimized for double-label experiments and a 63× oil immersion objective. Confocal microscopy was performed with a Zeiss Axioplan fluorescence microscope coupled with a Zeiss LSM510. Images were processed with Adobe Photoshop 7.0 software (San Jose, CA).

Apoptosis Assay

At 24 h after transfection, REF52.2 cells were fixed with formaldehyde and stained with the monoclonal anti-FLAG M2 antibody (Stratagene) and with Cy3-conjugated goat anti-mouse IgG (Dianova) and with DAPI to visualize nuclei. The percentage of apoptotic cells that showed fragmented nuclei, condensed chromatin, and membrane blebbing was determined among the transfected cells by fluorescence microscopy, counting 100–200 positive cells in each experiment. Data were collected from at least three independent experiments. Statistical significance was determined in a two-tailed Student's *t* test.

Immunoprecipitation

REF52.2 cells were transiently transfected as described above. For some experiments, cells were subjected to either serum starvation for 16 h, or 16-h serum starvation followed by 15 min 10 μM lysophosphatidic acid (LPA; Cayman Chemical, Ann Arbor, MI), or 10 μM forskolin (Sigma) for 15 min. Twenty-four hours after transfection, the cells were washed in ice-cold PBS and lysed in isotonic lysis buffer (10 mM $NaPO_4$, pH 8.0, 140 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% Nonidet-P40, and 50 μM leupeptin). The lysates were cleared by centrifugation and subjected to immunoprecipitation with the Par-4(P)T155-antibody (Pineda Antibody Service) or the mouse monoclonal anti-Par-4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), cross-linked to protein A or protein G agarose beads, at 4°C overnight. Immunoprecipitates were washed three times with lysis buffer and then subjected to SDS-PAGE according to standard protocols. For immunoprecipitation with the Par-4(P)T155-antibody, control experiments were performed using preimmune serum (Pineda Antibody Service).

Western Blot Analysis and Quantification of Par-4 Phosphorylation

Extract samples were separated on 10% SDS-PAGE (20 μg per lane) and electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Residual protein-binding sites on the membrane were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween. The membranes were incubated either with the mouse monoclonal anti-GFP antibody (Clontech) at 1:2000 dilution and the rabbit polyclonal anti-Par-4 antibody (Santa Cruz) at 1:2000 dilution. Bound antibodies were detected with peroxidase-conjugated secondary goat anti-mouse IgG or goat anti-rabbit at 1:5000 dilution (Dianova) using a SuperSignal West Pico chemoluminescence detection kit (Perbio Science, Bonn, Germany). Semiquantitative analysis of Par-4 phosphorylation was performed using densitometry. Par-4 bands after immunoprecipitation with the Par-4(P)T155 antibody were normalized to Par-4 bands in the respective input samples, and the phosphorylation level in unstimulated cells was defined as 100%. Significance was determined in a two-tailed paired Student's *t* test based on four independent experiments.

RESULTS

Par-4/Dlk–mediated Apoptosis Requires Binding of Dlk to Par-4 and Kinase Activity of Dlk

The direct interaction of Par-4 and Dlk depends on the C-terminal Par-4 leucine zipper and an arginine-rich region in the C-terminal domain of Dlk. In addition, the catalytic activity of Dlk seems to play an important role for Par-4/Dlk–mediated apoptosis (Page *et al.*, 1999a). These data suggest that the functional interplay of both proteins in apoptosis induction is influenced by phosphorylation events. In agreement with these observations, coexpression of GFP-tagged Par-4 and FLAG-tagged Dlk wt constructs (denoted Par-4 wt-GFP and FLAG-Dlk wt) in REF52.2 cells (Figure 1, a–d) resulted in a clear colocalization of both proteins at actin filaments (merged image Figure 1d) and the induction of apoptosis (condensed and fragmented nuclei Figure 1c). GFP-tagging neither affects Par-4 localization (Vetterkind *et al.*, 2005b) nor does the tag interfere with binding to known cellular ligands (Supplemental Figure S1, Supplemental Material). We next used the leucine zipper point mutant Par-4 L3-GFP (Boosen *et al.*, 2005) containing three exchanges of leucine residues to alanines in the coiled coil interface. As expected, this mutant failed to recruit FLAG-Dlk wt to the microfilament system (Figure 1, e–h) as indicated by the

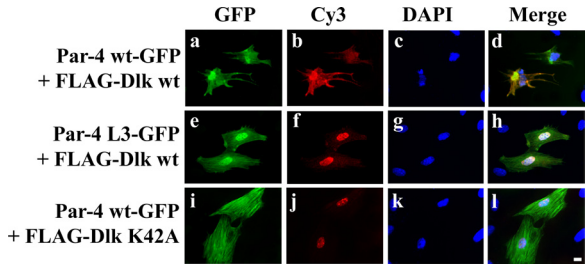

Figure 1. Dlk/Par-4 complex formation and catalytic activity of Dlk are essential for Dlk/Par-4–mediated apoptosis. REF52.2 cells were transiently transfected either with Par-4 wt-GFP/FLAG-Dlk wt (a–d), Par-4 L3-GFP/FLAG-Dlk wt (e–h) and Par-4 wt-GFP/FLAG-Dlk K42A (i–l). Twenty-four hours after transfection, the cells were fixed with formaldehyde and stained for indirect immunofluorescence microscopy with the monoclonal anti-FLAG M2 antibody and with Cy3-labeled secondary antibodies (b, f, and j). Nuclei were visualized with DAPI to analyze induction of apoptosis (c, g, and k). Scale bar, 10 μm.

prominent nuclear localization of the kinase (Figure 1f) and impaired induction of apoptosis (Figure 1g). Additionally, we analyzed a kinase-inactive point mutant, FLAG-Dlk K42A, which harbors an exchange of lysine to alanine at position 42 of rat Dlk (Kögel *et al.,* 1998). As shown previously (Page *et al.,* 1999a), the kinase-inactive mutant FLAG-Dlk K42A (Figure 1, i–l) remained in the nucleus and did not colocalize with Par-4 wt-GFP at actin filaments. Again, the prevention of Dlk/Par-4 complex formation resulted in impaired apoptosis induction. These analyses not only confirm that both, Dlk/Par-4 complex formation and the catalytic activity of Dlk are corequisites for Par-4/Dlk–mediated apoptosis, but also suggest that Par-4 may be an immediate downstream target of Dlk.

Par-4 Is a Candidate Substrate for Dlk

The minimal Dlk consensus motif conforms to the sequence R-X-X-(S/T) (Preuss *et al.,* 2003b). Because a C-terminal truncation mutant of Dlk has been shown to phosphorylate Par-4 in an in vitro phosphorylation assay (Page *et al.,* 1999a), we analyzed the rat Par-4 amino acid sequence for such motifs. Three potential Dlk phosphorylation sites could be identified, which are all located in the C-terminal half of the protein between the second nuclear localization signal (NLS2) and the death domain (Figure 2A): a single threonine residue at position 155 (T155) and two serine residues at positions 220 and 249 (Figure 2A). Because the consensus sequence comprising T155 also included a serine residue at position 154 (S154) and the required arginine residue in this area is surrounded by other basic residues, we also considered S154 as putative Dlk phosphorylation site. The Par-4 sequences were compared with those of known substrates of Dlk/ZIPK (Burch *et al.,* 2004), which all harbor an arginine residue at the −3 position preceding either a serine or threonine phospho-acceptor site, which are neighbored by polar rather than hydrophobic amino acid residues (Figure 2B). Because the same holds true for the putative phosphorylation sites on Par-4, we decided to analyze all of them in Dlk phosphorylation assays.

Dlk Phosphorylates Par-4 at Serine and Threonine Residues In Vitro

As an initial step in the identification of Par-4 phosphorylation sites we combined time-dependent in vitro phosphorylation assays with phosphoamino acid analyses (Figure 3). In addition, we included the catalytic subunit of PKA in our study, because it has been suggested that PKA is a candidate kinase for Par-4 phosphorylation in cancer cells (Gurumurthy *et al.,* 2005). Purified recombinant Par-4 protein (0.5–1 μg) was phosphorylated either by Dlk or PKA in the presence of [γ-^{32}P]ATP. Samples were taken after 1-, 5-, 10-, and 30-min incubation, and aliquots were analyzed by SDS-PAGE and autoradiography. As shown in Figure 3A, Par-4 was rapidly phosphorylated by recombinant Dlk with a faint band already visible after 1 min (Figure 3A, lane 2), which continuously increased in intensity resulting in a prominent phosphorylation after 30 min (Figure 3A, lane 5). A radioactive band at the position of Dlk (52 kDa) indicates that the kinase undergoes autophosphorylation. In a second set of experiments (Figure 3A, lanes 6–8) we included the recombinant kinase-inactive mutant Dlk K42A, which had been purified in a manner analogous to Dlk wt. Even after 30 min Par-4 was not phosphorylated when Dlk K42A was used in the phosphorylation assay (Figure 3A, lane 8), demonstrating that Par-4 phosphorylation in this assay was only dependent on recombinant Dlk kinase activity. In addition, no autophosphorylation was observed in contrast to Dlk wt in the absence (Figure 3A, lane 6) and presence (Figure 3A, lane 7) of Par-4. As expected, Par-4 could also be phosphorylated by PKA in vitro (Figure 3B, lanes 9–13). Subsequent phosphoamino acid analysis of the phosphorylated Par-4 protein bands revealed that Par-4 had been phosphorylated at threonine and serine residues by Dlk (Figure 3C) but only at serine residues by PKA (Figure 3D). The latter finding was surprising as it contradicted a previous report in which residue T155 of Par-4 had been suggested to be a phosphorylation site for PKA (Gurumurthy *et al.,* 2005).

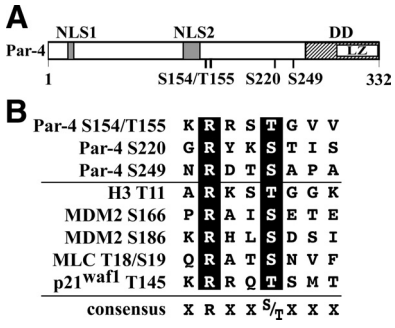

Figure 2. (A) Schematic representation of potential Dlk phosphorylation sites in the Par-4 protein. Full-length rat Par-4 (Par-4 wt, 332 amino acids) contains two nuclear localization signals (NLS1 and NLS2), and a leucine zipper motive (LZ) located within the death domain (DD) at the carboxy-terminus (shaded box). The putative Dlk phosphorylation sites in the rat Par-4 protein are indicated. (B) Sequence comparison of known Dlk substrates. The positions of the required arginine residue at the −3 position preceding the phospho-acceptor site are highlighted in all substrates, suggesting a minimal Dlk consensus motif of R-X-X-(S/T).

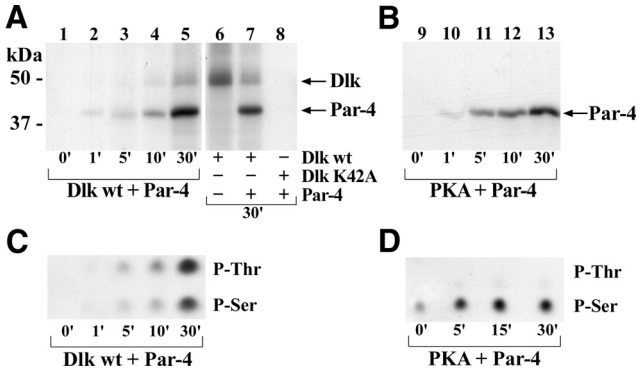

Figure 3. Time-dependent in vitro phosphorylation of recombinant rat Par-4 by Dlk and PKA. Purified recombinant Par-4, 0.5–1 μg, was phosphorylated by Dlk (A and C) or with the catalytic subunit of PKA (B and D) in the presence of [γ-^{32}P]ATP. Phosphorylation was monitored over a period of 30 min. (A and B) Autoradiography of a 10% SDS-PAGE. Note that phosphate incorporation constantly increases over time (lanes 1–5). For Dlk (A) phosphorylation of Par-4 was also analyzed for the kinase-dead mutant Dlk K42A in comparison to wild-type protein (lanes 6–8). (C and D) Phosphoproteins were blotted onto nitrocellulose membrane, isolated, and subjected to acid hydrolysis. Phosphoamino acids were separated by electrophoresis on thin-layer cellulose plates and visualized by autoradiography. Note that Dlk phosphorylated Par-4 at threonine and serine residues, whereas PKA phosphorylated Par-4 only at serine residues.

Phosphorylation of Par-4 at T155 Is Essential for Par-4/Dlk–mediated Apoptosis

For the next step aiming toward the identification of in vivo Dlk phosphorylation site(s) in Par-4, we generated four

Par-4 alanine mutants, each of which carried a single mutation in one of the putative phosphorylation sites. In these mutants, designated S154A, T155A, S220A, and S249A, the corresponding serine or threonine residue had been substituted by alanine. The mutants were expressed as C-terminally GFP-tagged fusion proteins in REF52.2 cells and analyzed for their subcellular distribution by fluorescence microscopy (Supplemental Figure S2, Supplemental Material). The single amino acid substitutions did not affect the association with the microfilament system, nor did the overexpression of Par-4 wt or any of the Par-4 mutants alone induce apoptosis in the absence of coexpressed Dlk. We next assayed the ability of these mutants to induce apoptosis in conjunction with Dlk (Figure 4). REF52.2 cells were cotransfected with FLAG-Dlk and either C-terminally GFP-tagged Par-4 S154A, T155A, S220A, or S249A or as a control with Par-4 wt. Twenty-four hours after transfection, the cells were fixed and stained with the monoclonal FLAG antibody M2 to visualize Dlk and with DAPI to analyze nuclei (Figure 4A). Replacement of any of the three serine residues by alanine had no influence on Dlk recruitment and induction of apoptosis: Coexpression of S154A (Figure 4A, e–h), S220A (Figure 4A, m–p), and S249A (Figure 4A, q–t) with FLAG-Dlk resulted in translocation of Dlk from the nucleus to actin filaments and to the induction of apoptosis similar to the Par-4 wt control (Figure 4A, a–d). In contrast, when Dlk was coexpressed with the Par-4 mutant T155A (Figure 4A, i–l), kinase translocation was impaired as shown by prominent retention of the protein in the nuclei of the cells (Figure 4Aj), whereas Par-4 T155A was clearly associated with the microfilament system (Figure 4Ai). Concomitantly, no reorganization of the actin cytoskeleton occurred and hardly any apoptosis induction could be observed by DAPI staining (Figure 4Ak).

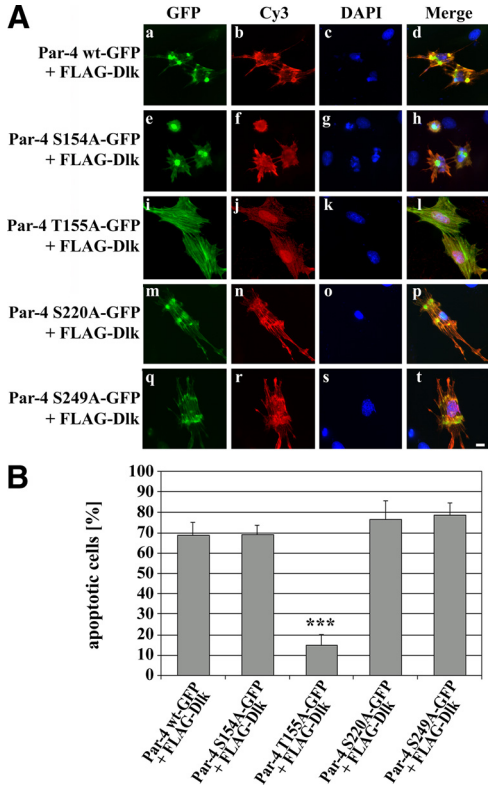

Figure 4. Coexpression of Par-4 phospho-mutants and Dlk in rat fibroblasts. (A) REF52.2 cells were cotransfected with FLAG-Dlk and either Par-4 wt-GFP (a–d), Par-4 S154A-GFP (e–h), Par-4 T155A-GFP (i–l), Par-4 S220A-GFP (m–p), and Par-4 S249A-GFP (q–t). At 24 h after transfection, the cells were fixed with formaldehyde and stained for indirect immunofluorescence microscopy with the monoclonal anti-FLAG M2 antibody and anti-mouse IgG-Cy3 (b, f, j, n, and r) and with DAPI to analyze induction of apoptosis (c, g, k, o, and s). Note that after coexpression of Dlk and Par-4 phospho-mutant T155A the kinase was only partially recruited to actin filaments and was mainly localized in the nucleus of the cotransfected cells. Scale bar, 10 μm. (B) Quantitative analysis of apoptosis induction in REF52.2. FLAG-Dlk and the C-terminally GFP-tagged Par-4 constructs Par-4 wt, S154A, T155A, S220A, and S249A were coexpressed in REF52.2 cells. Twenty-four hours after transfection, the cells were fixed with formaldehyde and stained with the anti-FLAG M2 antibody and with DAPI to visualize nuclei. The percentage of cells that showed apoptotic morphology (i.e., fragmented nuclei, condensed chromatin and membrane blebbing) was determined among the cotransfected cells by fluorescence microscopy, counting 100–200 positive cells in each experiment. The graph represents the mean value from three independent experiments; error bars, SD (*** $p < 0.001$).

The efficacy of each Par-4 construct in apoptosis induction was estimated from the percentage of apoptotic cells showing fragmented nuclei, condensed chromatin and membrane blebbing among the Par-4/Dlk coexpressing cells (Figure 4B). This analysis revealed apoptosis induction of ~15% in T155A/Dlk coexpressing cells, which was significantly lower than apoptosis induction in cells coexpressing Par-4 wt and Dlk (~69% apoptotic cells) or any of the other Par-4 mutants (~69% for S154A/Dlk, 76% for S220A/Dlk, and 79% for S249A/Dlk). These data demonstrate that phosphorylation of Par-4 at T155 is crucial for Dlk/Par-4–mediated apoptosis in rat fibroblasts.

T155 Is the Preferred Residue Phosphorylated by Dlk In Vitro

Because apoptosis induction by Par-4 and Dlk strongly depended on Par-4 phosphorylation at T155 (Figure 4) as well as catalytically active Dlk (Figure 1), mainly phosphorylating threonine residues in vitro (Figure 3C), we next analyzed whether Par-4 was indeed phosphorylated by Dlk at residue T155. Therefore, recombinant Par-4 proteins and Dlk were used in in vitro phosphorylation assays as already described above. Samples were subjected to SDS-PAGE and subsequent autoradiography (Figure 5, top panel). Although Par-4 wt and mutant Par-4 S154A were phosphorylated to similar extents (Figure 5, top panel, cf. lanes 1 and 2), only a faint radioactive band was observed for the Par-4 T155A mutant (Figure 5, top panel, lane 3), demonstrating that T155A was in fact the main site phosphorylated by Dlk in vitro. Phosphate incorporation was even further reduced in the Par-4 S154A/T155A double mutant (Figure 5, top panel, lane 4), suggesting some phosphorylation of S154 in the T155A mutant, even though this serine residue is not located in a perfect Dlk consensus sequence. The Coomassie staining of the same gel is shown in the bottom panel of Figure 5, demonstrating equal protein load.

Phosphorylation of T155 by Dlk In Vivo is a Key Event in Dlk/Par-4–induced Apoptosis

For direct analysis of Par-4 T155 phosphorylation in vivo, we first raised a polyclonal antibody against the synthetic phospho-peptide KRRSpTGVVN corresponding to residues 151–159 of the rat Par-4 sequence containing phosphothreonine [denoted Par-4(P)T155]. The phospho-specific Par-4(P)T155 antibody did not bind to denatured phosphorylated Par-4 protein in Western blot analyses, but was functional in immunoprecipitation and immunofluorescence experiments (Figure 6). The specificity of the Par-4(P)T155

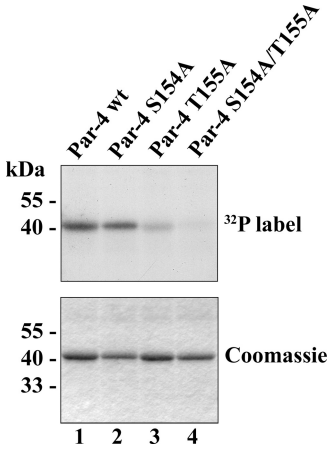

Figure 5. In vitro phosphorylation of Par-4 wt and different Par-4 phospho-mutants by Dlk. Purified recombinant Par-4 wt (2 μg, lane 1), Par-4 S154A (lane 2), Par-4 T155A (lane 3), and Par-4 S154A/T155A (lane 4) were phosphorylated by recombinant Dlk in the presence of [γ-^{32}P]ATP, analyzed on 10% SDS-PAGE, and visualized by autoradiography (top). Equal protein loading of all Par-4 constructs was demonstrated by Coomassie staining of the same gel (bottom). For better depiction of differences in signal intensities a shorter exposure time was chosen for autoradiography than in Figure 3. Hence autophosphorylation of Dlk is not visible.

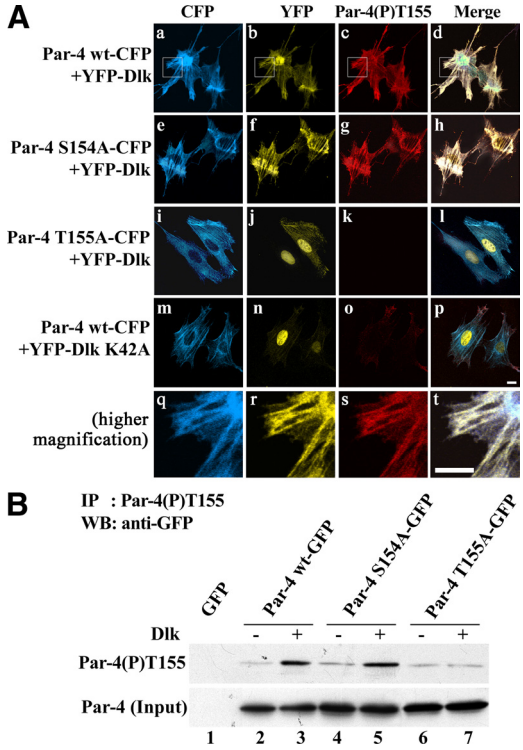

Figure 6. The Par-4(P)T155 antibody recognizes Par-4 when phosphorylated by Dlk at T155. (A) REF52.2 cells were cotransfected with YFP-Dlk and either Par-4 wt-CFP (a–d), Par-4 S154A-CFP (e–h), or Par-4 T155A-CFP (i–l). As a control, REF52.2 cells were also transfected with Par-4 wt-CFP and the kinase-inactive mutant Dlk K42A (m–p). Twenty-four hours after transfection, the cells were fixed with formaldehyde and stained with the rabbit polyclonal Par-4(P)T155 antibody and anti-rabbit IgG-Cy3 (c, g, k, and o). The phospho-specific Par-4(P)T155 antibody failed to stain cells either coexpressing YFP-Dlk/Par-4 T155A-CFP or Par-4 wt-CFP/YFP-Dlk K42A, suggesting phosphorylation at T155 by Dlk. At higher magnification (q–t) of the insets marked in (a–d) the colocalization of Dlk (r) and phosphorylated Par-4 (s) becomes more evident. Note that both analyses show a slightly punctate staining pattern that matches in the merged image (t). Scale bar, 10 μm. (B) Immunoprecipitation of Par-4 with the phospho-specific antibody Par-4(P)T155. REF52.2 cells were transfected either with GFP-vector, Par-4 wt-GFP, Par-4 S154A-GFP, or Par-4 T155A-GFP alone (lanes 1, 2, 4, and 6, respectively) or cotransfected with the same Par-4 constructs and FLAG-Dlk (lanes 3, 5, and 7). Twenty-four hours after transfection, cell extracts were prepared and subjected to immunoprecipitation with the rabbit polyclonal Par-4(P)T155 antibody. The proteins were separated by SDS-PAGE and analyzed by Western blotting with the monoclonal anti-GFP antibody. Note that in conjunction with Dlk only Par-4 wt-GFP (lane 3) and Par-4 S154A-GFP (lane 5) were precipitated with the anti-Par-4(P)T155 antibody but not Par-4 T155A-GFP (lane 7). The input (bottom) represents 20 μg of whole-cell lysate, which was simultaneously subjected to SDS-PAGE and Western blot analysis with the anti-GFP antibody.

antibody was tested in a blocking approach with the phosphopeptide KRRSpTGVVN (Supplemental Figure S3, Supplemental Material). To analyze Dlk, Par-4, and T155 phosphorylation in the same cell, we performed cotransfection experiments using YFP-tagged Dlk constructs in combination with C-terminally CFP-tagged Par-4 constructs. Twenty-four hours after transfection, REF52.2 cells were fixed, stained with the polyclonal Par-4(P)T155 antibody and a Cy3-conjugated secondary antibody, and inspected by confocal microscopy (Figure 6A). In cells coexpressing YFP-Dlk and Par-4 wt-CFP (Figure 6A, a–d) colocalization of both constructs was observed at the microfilament system, resulting in dramatic reorganization indicative of apoptosis (merged image in Figure 6Ad, cf. also Figure 4). In addition, indirect immunofluorescence staining with the Par-4(P)T155 antibody revealed a prominent staining of the coexpressing cells (Figure 6Ac), demonstrating Par-4 phosphorylation at residue T155 in vivo. At higher magnification the colocalization (Figure 6A, q–t) of YFP-Dlk and phosphorylated Par-4 could be demonstrated revealing a slightly punctate pattern. The same results were obtained in cells expressing a combination of YFP-Dlk and Par-4 S154A-CFP (Figure 6A, e–h). Again, phosphorylation of residue T155 was indicated by staining the cells with the Par-4(P)T155 antibody (Figure 6g). As expected, antibody Par-4(P)T155 did not recognize the Par-4 T155A-CFP mutant (Figure 6Ak). In agreement with our previous analyses, Par-4 T155A-CFP was still associated with actin filaments (cf. Figure 6Ai with Supplemental Figure S2i) but was markedly impaired in recruiting YFP-Dlk to the actin cytoskeleton, resulting in prominent nuclear localization of Dlk (Figure 6Aj). This effect was more pronounced in this experiment compared with the analogous transfection experiments employing a Dlk-FLAG construct (cf. Figures 6Aj and 4Aj). This discrepancy can be explained by the signal enhancement achieved by indirect immunofluorescence using polyclonal secondary antibodies compared with direct visualization by YFP. The strongest evidence that Dlk indeed phosphorylated Par-4 at T155 in this experimental setup was obtained by expressing Par-4 wt-CFP in conjunction with the kinase-inactive mutant YFP-Dlk K42A (Figure 6A, m–p). As expected from our initial experiments (Figure 1), Dlk mutant K42A was not recruited to the microfilament system but mainly remained in the nucleus. Furthermore, the signal obtained from staining the cells with the Par-4(P)T155 antibody was almost reduced to background levels when compared with the signal obtained from cells coexpressing wild type YFP-Dlk and Par-4 wt-CFP (Figure 6Ac). The weak staining observed with the Par-4(P)T155 antibody in REF52.2 cells coexpressing Par wt-GFP and the kinase-

dead Dlk mutant (Figure 6Ao) may reflect basal phosphorylation of ectopically expressed Par-4 by endogenous Dlk. In agreement with this assumption, phosphorylation of ectopically (and endogenous) Par-4 protein by endogenous kinase activity could be demonstrated in immunoprecipitates from REF52.2 cells expressing Par-4 wt-GFP only (Supplemental Figure S1B, Supplemental Material).

Immunoprecipitation analyses provided further evidence that Par-4 was indeed phosphorylated at T155 by Dlk in vivo (Figure 6B). Three GFP-tagged Par-4 constructs (Par-4 wt-GFP, Par-4 S154A-GFP, and Par-4 T155A-GFP) were expressed in REF52.2 cells either alone or in combination with FLAG-Dlk wt, and as a control GFP was expressed alone. Twenty-four hours after transfection, the cells were harvested, and the cell lysates were subjected to immunoprecipitation with the Par-4(P)T155 antibody and analyzed by SDS-PAGE and Western blotting. As shown in Figure 6B (top row) the Par-4(P)T155 antibody precipitated a single protein band of ~70 kDa corresponding to the GFP-tagged Par-4 proteins from lysates of cells cotransfected with Par-4 wt/Dlk (Figure 6B, lane 3) and Par-4 S154A/Dlk (Figure 6B, lane 5), but not from lysates derived from cells that had been transfected either with Par-4-GFP wt or Par-4 S154A-GFP alone (Figure 6, lanes 2 and 4, respectively). No protein was immunoprecipitated from Par-4 T155A-GFP/Dlk cotransfected cells (Figure 6, lane 7) and from cells expressing either Par-4 T155A-GFP or the GFP-vector alone (Figure 6, lanes 6 and 1, respectively). Similar expression levels of the Par-4 constructs were confirmed by SDS-PAGE and Western blotting with a GFP antibody analyzing 20 μg of total protein of each cell lysate (Figure 6B, bottom row).

The Cellular Effect of T155 Phosphorylation Cannot Be Mimicked by Acidic Amino Acids

A common strategy of mimicking constitutive phosphorylation of a protein is the substitution of serine and threonine residues by acidic amino acids. We thus generated C-terminally GFP-tagged phospho-mutants of Par-4 carrying either an exchange of an aspartic acid residue or a glutamic acid residue at position 155 of the rat Par-4 amino acid sequence (denoted Par-4 T155D-GFP or Par-4 T155E-GFP, respectively). Hence, we tested both phospho-mutants for induction of apoptosis in conjunction with Dlk in REF52.2 cells (Figure 7). Both Par-4 phospho-mutants clearly colocalized with the actin filament system (Figure 7, a and e). However, both Par-4 mutants failed to recruit the coexpressed FLAG-Dlk construct, which was mainly retained in the nucleus of the cotransfected cells (Figure 7, b and f). Furthermore, no induction of apoptosis was observed. Thus introducing acidic amino acids at position 155 in the rat Par-4 amino acid sequence at residue T155 is not sufficient to trigger Par-4/Dlk–induced apoptosis.

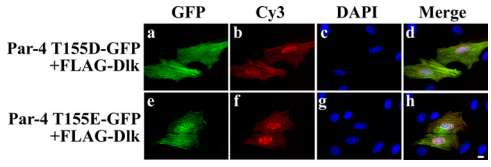

Figure 7. Introduction of acidic amino acids cannot mimic phosphorylation of Par-4 at T155. REF52.2 cells were cotransfected either with FLAG-Dlk and the GFP-tagged Par-4 phospho-mutant T155D (a–d) or with FLAG-Dlk and the GFP-tagged phospho-mutant T155E (e–h). Twenty-four hours after transfection, the cells were fixed and stained for indirect fluorescence microscopy with the monoclonal anti-FLAG M2 antibody and with Cy3-labeled secondary antibodies (b and f). Nuclei were visualized with DAPI to analyze induction of apoptosis (c and g). Scale bar, 10 μm.

Phosphorylation of Endogenous Par-4 at T155 Is Enhanced after LPA Stimulation

Because all experimental data so far had been based on overexpression experiments, we finally investigated whether endogenous Par-4 was also phosphorylated at threonine-155 in response to Dlk activation (Figure 8). Although phosphorylation at T155 could not satisfactorily be depicted in immunofluorescence studies (our unpublished observations), the higher sensitivity of immunochemical detection after immunoprecipitation in immunoblots allowed the detection of endogenous Par-4 phosphorylated at T155, indicating that the phosphorylation event was not restricted to ectopically expressed Par-4 wt-GFP (Figure 8 and Supplemental Figure S1B, Supplemental Material). Surprisingly, we first noticed that even in untreated Ref 52.2 cells there was a basal level of Par-4 phosphorylation at T155 (Figure 8A, lane 2). We therefore decided against classical inductors of apoptosis like ionomycin or thapsigargin, which both lead to an increase in Par-4 expression (Sells *et al.*, 1994; Hsu *et al.*, 2002), thus complicating normalization of the phosphorylation status with respect to total Par-4 levels. It has recently been shown that Dlk becomes activated by ROCK via phosphorylation at threonine-265 (Hagerty *et al.*, 2007), a conserved phosphorylation site that is critical for kinase activity (Sato *et al.*, 2006). Consequently, treatment with the Rho/ROCK activator LPA eventually leads to Dlk activation. Hence REF52.2 cells were serum-starved for 16 h and then treated with LPA for 15 min (Figure 8A, lane 4). These cells were

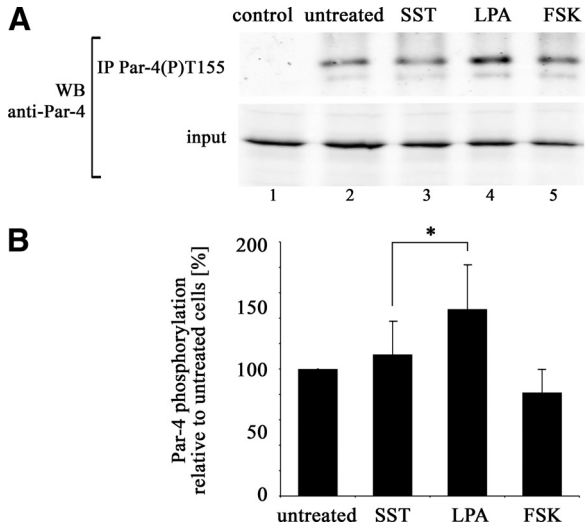

Figure 8. Phosphorylation of endogenous Par-4 protein in REF52.2 cells. (A) Immunoprecipitation of endogenous Par-4 with the phospho-specific antibody Par-4(P)T155 (top). Cells were left untreated (lane 2), serum-starved for 16 h (SST, lane 3), stimulated with 10 μM LPA lysophosphatidic acid for 15 min after serum starvation (LPA, lane 4), or treated with 10 μM forskolin for 15 min (FSK, lane 5). As control for antibody specificity the IP on untreated cells was also performed with preimmune serum (lane 1). Immunoprecipitates were analyzed by Western blot analysis with the mouse monoclonal anti-Par-4 antibody. The input (bottom) represents 20 μg of whole-cell lysate. (B) Densitometric analysis of four independent experiments. Par-4 bands after immunoprecipitation with the Par-4(P)T155 antibody were normalized to Par-4 bands in the respective input samples, and the phosphorylation level in unstimulated cells was defined as 100% (* $p < 0.05$).

compared with untreated cells (Figure 8A, lane 2), and samples exposed to serum starvation only (Figure 8A, lane 3) and to cultures that had been treated with forskolin to increase endogenous cAMP levels to activate PKA (Figure 8A, lane 5). None of the cultures underwent apoptosis, as judged by immunofluorescence analyses (data not shown). Cell lysates were subjected to immunoprecipitation with the phospho-specific Par-4(P)155 antibody and the immunoprecipitated protein was compared with total Par-4 expression levels (Figure 8, input in bottom row). Neither serum starvation alone nor PKA activation by forskolin led to an increase in Par-4 phosphorylation at T155. In contrast, a significantly higher level of endogenous Par-4 phosphorylation was achieved by serum starvation in combination with LPA treatment as revealed by densitometric analysis of four independent experiments (Figure 8B), suggestive of enhanced phosphorylation by Dlk. Unfortunately, there are currently no specific inhibitors of Dlk available allowing clear distinction between the effect of ROCK and Dlk. Therefore at present we can only speculate that endogenous Par-4 is phosphorylated at threonine-155 by Dlk after LPA stimulation.

Taken together, our data reveal a mutual functional relationship between the phosphorylation of Par-4 by Dlk and Dlk/Par-4 association indicating that the posttranslational modification of Par-4 results in stabilization of Dlk/Par-4 complexes. The latter is clearly dependent on Dlk activity and does not simply require the introduction of negative charges, because both Par-4 phosphorylation mutants fail to recruit Dlk to the microfilament system. Future research will have to elucidate how Dlk/Par-4 interaction is enhanced by phosphorylation at the molecular level.

DISCUSSION

Previous investigations have shown that Par-4–mediated induction of apoptosis in rat fibroblasts critically depends on the protein's capacity to recruit active Dlk to the microfilament system, mediating cytoskeletal changes via MLC phosphorylation (Vetterkind *et al.,* 2005b). In the present study we show that Par-4 itself is an in vivo downstream target of Dlk and that enhanced Par-4 phosphorylation by Dlk at T155 is an important step for promoting apoptosis in REF52.2 cells. The importance of T155 phosphorylation in Par-4–mediated apoptosis is corroborated by earlier investigations on the role of Par-4 in apoptosis induction in cancer cells (Gurumurthy *et al.,* 2005). In contrast to our findings, however, the authors suggested PKA to be responsible for T155 phosphorylation as PKA activity was significantly enhanced in the prostate cancer cell lines investigated. In addition, using NIH3T3/Ras transformed cells in transfection experiments they showed that in vivo phosphorylation of a Par-4 deletion construct (137–187) was abolished upon addition of the PKA inhibitory peptide PKI. In vitro phosphorylation of immunoprecipitated Par-4 constructs by recombinant PKA also revealed a significant decrease in phosphate incorporation into a T155A mutant in the same study (Gurumurthy *et al.,* 2005). The latter finding clearly contradicts our own in vitro phosphorylation analyses using recombinant proteins only. In contrast to recombinant Dlk, which preferentially phosphorylated Par-4 at T155, phosphoamino acid analyses revealed that PKA only phosphorylated serine residues on recombinant Par-4 protein, suggesting that T155 is not an in vitro target for PKA. In addition, neither the application of a PKA inhibitor (unpublished observations) nor stimulation of PKA via forskolin (Figure 8) had any effect on T155 phosphorylation in rat fibroblasts. Different cell types (e.g., prostate cancer cells and rat fibroblasts) may exhibit different kinase activities leading to Par-4 phosphorylation at T155, thus explaining how modulating PKA activity may affect Par-4 phosphorylation in one cell type but not the other. Furthermore, Dlk/Par-4 complex formation may not occur to similar extents in all cell types and species (Shoval *et al.,* 2007 and see below). However, the discrepancies regarding the in vitro phosphorylation data cannot satisfactorily explained at present. In any case, it is clear that in REF52.2 cells, Dlk and not PKA is the kinase responsible for Par-4 phosphorylation at T155 leading to the induction of apoptosis in these cells.

Interestingly, the phosphorylation event seems to be intimately correlated with Dlk recruitment to the microfilament system, which is an important step during Par-4 apoptosis induction in rat fibroblasts (Vetterkind *et al.,* 2005b). The cytoskeletal association is also indicative of Dlk/Par-4 complex formation, even though at present it remains unclear whether this association occurs prior or at the same time with actin filament binding. In our study, complex formation was clearly impaired, if either a kinase-dead Dlk mutant was used or T155 was mutated into a nonphosphorylatable residue. In both cases, a prominent nuclear localization was observed for Dlk proteins, whereas the Par-4 constructs always associated with cellular stress fibers. In this context, it is important to note that a decrease in Dlk/Par-4 complex formation and consequently significantly lower apoptosis induction was observed for any mutation of T155, irrespective of whether T155 had been mutated into alanine or into acidic residues, mimicking dephosphorylated and phosphorylated Par-4 proteins, respectively. These findings suggest that the phosphorylation event is coupled to the stabilization of the Par-4/Dlk complex and association with the actin cytoskeleton. The exact molecular mechanism how T155 phosphorylation of Par-4 may contribute to complex stability remains to be elucidated, but it is an attractive model allowing prolonged anchorage of the kinase to the actin cytoskeleton, which could support the cytoskeletal reorganization during apoptosis, e.g., leading to a more efficient phosphorylation of MLC.

Stable Dlk/Par-4 complexes may also explain how Dlk compensates for the lack of a death domain and other structural features present in the related family member DAPK: Even though Dlk and DAPK show high sequence homology in the N-terminal kinase domains, they both significantly differ in size and domain structure in the extracatalytic domains. The 160-kDa protein DAPK harbors a Ca^{2+}/CaM-binding autoregulatory domain, eight ankyrin repeats, two P-loop motifs followed by a cytoskeletal binding region, and a C-terminal death domain (Bialik and Kimchi, 2006). The ankyrin repeats and the cytoskeletal binding region have been shown to direct DAPK to the actin cytoskeleton, whereas the death domain is an important regulator of DAPK activity. In contrast, the extracatalytic region of Dlk neither contains any element allowing for cytoskeletal association nor does it harbor a death domain. Instead, Dlk contains two NLSs), an arginine-rich domain (amino acids 338–417) mediating the binding to Par-4 (Page *et al.,* 1999a), and a leucine zipper mediating homodimerization and interactions with other partners (Page *et al.,* 1999b; Engemann *et al.,* 2002). Because Par-4 binds to actin filaments and contains a C-terminal death domain, the Dlk/Par-4 complex can be thought of as a bipartite structural mimic of DAPK (Bialik and Kimchi, 2006). Stabilization of this complex through T155 phosphorylation by Dlk would thus clearly be advantageous for apoptosis induction. A potential stabilizing effect of T155 phosphorylation on Dlk/Par-4 complexes would also be beneficial in other cell systems where Dlk and

Par-4 have been suggested to exert their proapoptotic effects in the nucleus. Finally, there is even functional evidence suggesting that Dlk/Par-4 complexes exhibit similar properties as compared with DAPK. For the latter it has been shown that the isolated death domain acts as a dominant negative mutant when expressed in eukaryotic cells (Cohen *et al.*, 1999; Raveh *et al.*, 2000), whereas deletion of this region may attenuate the kinase's proapoptotic activity (Cohen *et al.*, 1999; Kuo *et al.*, 2003). In analogy, studies have demonstrated that overexpression of the Par-4 leucine zipper domain partially overlapping with the protein's death domain also prevents apoptosis induction in a dominant negative manner (Sells *et al.*, 1997). Deletion of the leucine zipper domain abrogates the proapoptotic function of Par-4, suggesting that this domain is essential for the proapoptotic activity of the protein (Sells *et al.*, 1997). In REF52.2 cells the C-terminus of Par-4 (aa 266–332) does not bind to actin filaments, both in vitro and vivo (Vetterkind *et al.*, 2005b) and thus fails to recruit Dlk to the microfilament system, leading to a decrease in apoptosis. For both scenarios it is fair to assume that the C-terminal domains of the death-inducing proteins DAPK and Par-4 compete with endogenous DAPK and Dlk/Par-4 complexes, respectively, by sequestering apoptosis-relevant interaction partners.

It has recently been shown that murine Dlk but not the human orthologue ZIPK directly binds to Par-4 (Shoval *et al.*, 2007). The authors speculate that the cytoplasmic retention of murine Dlk by Par-4 compensates for the evolutionary loss of threonine 299, a highly conserved phosphorylation site in all nonmurine species investigated so far. Phosphorylation at threonine-299 is crucial for cytoplasmic localization of ZIPK (Graves *et al.*, 2005). The loss of threonine-299 in murine Dlk not only explains the predominant nuclear localization of Dlk in the absence of sufficiently high Par-4 concentrations, but also the need for a different regulatory mechanism mediating cytoplasmic retention of Dlk. In general, murine Dlk shows a much higher sequence divergence from the human orthologue ZIPK in the extra-catalytic domain than any other ZIPK protein, even from more distantly related vertebrates (Shoval *et al.*, 2007). This sequence diversity also accounts for the Par-4 binding property of Dlk but not ZIPK. Whether this interaction is indeed a unique feature of murine rodents remains to be shown. At least in ferrets, for which the protein sequence of Dlk is not known yet, Dlk was shown to colocalize with endogenous Par-4 on actin filaments in differentiated vascular smooth muscle cells (Vetterkind and Morgan, 2009). Even in human ARPE19 cells that have a much more pronounced microfilament system than the HeLa cells investigated (Shoval *et al.*, 2007), an association of ZIPK with the microfilament system was observed (Takamoto *et al.*, 2006). Taken together, these data suggest that in different species ZIPK protein recruitment to the microfilament system is achieved by different proteins: Although Par-4 functions as the predominant recruitment factor for Dlk in murine cells, human ZIPK may be targeted to microfilaments via different proteins, yet to be identified. The outcome, however, is the same in both cases: a stable association of either kinase with a microfilament-associated protein eventually leads to the phosphorylation of MLC, the regulatory light chain of myosin II, responsible for inducing major cytoskeletal changes during apoptosis (Murata-Hori *et al.*, 2001; Vetterkind *et al.*, 2005b).

At first glance, a quite unexpected finding was the basal phosphorylation level of endogenous Par-4 at T155 in untreated Ref52.2 cells, a phenomenon not observed when analyzing ectopically expressed Par-4 constructs in the absence of exogenous Dlk (Figure 6B). In addition, neither basal phosphorylation of overexpressed Par-4 wt-CFP (Figure 6Ao) nor enhanced phosphorylation of endogenous Par-4 at T155 after serum starvation and LPA treatment lead to the induction of apoptosis (Figure 8 and data not shown). However, these findings are in good agreement with several previous observations: 1) Dlk/Par-4–containing complexes have been shown to facilitate contraction in differentiated vascular smooth muscle cells by regulating MLC phosphorylation, indicating that both proteins may also function as physiological modulators of the microfilament system (Vetterkind and Morgan, 2009). 2) Neither Dlk nor Par-4 overexpression alone activates apoptotic pathways in rodent fibroblasts (Kögel *et al.*, 1999; Page *et al.*, 1999a; Gurumurthy *et al.*, 2005), suggesting that endogenous expression levels in nonapoptotic fibroblasts do not suffice to generate high enough concentrations of Dlk/Par-4 complexes, allowing initiation of apoptosis. 3) Apoptosis induction by ionomycin (Sells *et al.*, 1994) or thapsigargin (Hsu *et al.*, 2002) is accompanied by an increase in Par-4 expression. Taken together, these findings suggest a certain threshold in Dlk/Par-4 complex formation that needs to be overcome for apoptosis induction. Alternatively, additional phosphorylation of Par-4 at sites other than T155 may further influence the proapoptotic function of Dlk/Par-4 complexes (see below).

So far, we have only discussed T155 phosphorylation with respect to its effect on Par-4 association with Dlk. However, phosphorylation might be a more general regulatory mechanism in regulating Par-4–mediated apoptosis (Goswami *et al.*, 2005; Gurumurthy *et al.*, 2005). Although prominent T155 phosphorylation clearly promotes apoptosis in both, REF52.2 fibroblasts (this report) and cancer cells (Gurumurthy *et al.*, 2005), phosphorylation at serine-249 by Akt, a key cell survival kinase has been reported to attenuate Par-4–induced apoptosis (Goswami *et al.*, 2005). Again, these studies were performed in cancer cell lines where nuclear translocation of Par-4 is crucial for apoptosis induction. Here the authors suggested that the anti-apoptotic effect of Akt on Par-4 is based on the interaction of phosphorylated Par-4 with 14-3-3 proteins, which are well established as molecular sequestration sites for cell death promoters. Binding to 14-3-3 leads to Par-4 retention within the cytoplasm, thus preventing the induction of apoptosis. Interestingly, Par-4 phosphorylation by Akt also depends on both, kinase activity of Akt and the presence of the S249 phosphorylation site suggesting that, again, phosphorylation is coupled to stable complex formation.

Taken together, these data reveal that Par-4 is an in vivo substrate for proapoptotic (Dlk) as well as antiapoptotic (Akt) kinases and that Par-4 phosphorylation leads to the formation and/or stabilization of protein complexes either initiating apoptosis or promoting cell survival. Hence, Par-4 could function as a molecular switch between these two cell fates by reflecting the endogenous balance between proapoptotic and antiapoptotic signals through its phosphorylation state.

ACKNOWLEDGMENTS

We thank Reinhild Brinker for excellent technical assistance and Philipp Wörsdörfer for testing the PKA inhibitor. This study was supported by the Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung (106365) to U.P.

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