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ERK5 pathway regulates the phosphorylation of tumour suppressor hDlg during mitosis

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ABSTRACT

Human disc-large (hDlg) is a scaffold protein critical for the maintenance of cell polarity and adhesion. hDlg is thought to be a tumour suppressor that regulates the cell cycle and proliferation. However, the mechanism and pathways involved in hDlg regulation during these processes is still unclear. Here we report that hDlg is phosphorylated during mitosis, and we establish the identity of at least three residues phosphorylated in hDlg; some are previously unreported. Phosphorylation affects hDlg localisation excluding it from the contact point between the two daughter cells. Our results reveal a previously unreported pathway for hDlg phosphorylation in mitosis and show that ERK5 pathway mediates hDlg cell cycle dependent phosphorylation. This is likely to have important implications in the correct timely mitotic entry and mitosis progression.

1. Introduction

Human discs-large (hDlg) protein, also called Dlg1, dlgh1 and SAP97, is an orthologue of the *Drosophila* tumour suppressor Dlg. It is a member of the membrane-associated guanylate kinase family of scaffold proteins, which include the post-synaptic density protein-95 (PSD95), the zonula occludens proteins (ZO1, ZO2 and ZO3) or the calcium/calmodulin dependent serine protein kinase (CASK). hDlg has multiple protein–protein interaction domains such as three PDZ domains, a SH3 domain, an L27 domain and a catalytically inactive guanylate kinase (GUK)-like region [1]. Functions of hDlg have been related to growth control, and to the establishment and maintenance of cell polarity and cell adhesion [2–4]. Moreover, gene-targeted mice lacking full-length hDlg showed defects in the morphogenesis of the kidney and urogenital tracts [5,6].

Several studies have shown that either inactivation or depletion of the *Drosophila* Dlg protein results in neoplastic growth of imaginal disc epithelial cells [7]. Moreover, the expression of the Dlg mammalian counterpart (hDlg) in epithelial-derived cancers (such as cervical, gastric and colon cancers) is extremely low or even absent [8]. hDlg have been shown to interact with a number of proteins related to the cell cycle control. Thus, hDlg binds to the tumour suppressor adenomatous polyposis coli (APC) and negatively regulates cell cycle progression from G1 to S phase [9]. Also, hDlg interacts with several viral oncoproteins, such as the viral human papillomavirus (HPV) E6, the human T cell leukaemia virus type 1 (HTLV-1) Tax or the adenoviral E4 ORF1 [10]. In addition, the association of these viral proteins with hDlg regulates their oncogenic activity [10], although the mechanism by which hDlg complexes are regulated in the cell cycle is still unclear. In this regard, it has been shown that binding of HPV E6 protein to hDlg causes a decrease in hDlg protein levels by inducing its proteasome-mediated degradation [11]. In epithelial cell lines, the phosphorylation of hDlg makes it more susceptible to degradation induced by the HPV E6 [12].

Besides the indication that hDlg phosphorylation may modulate its protein levels in cells, during the last few years phosphorylation has been established as a mechanism for regulating hDlg functions and its localisation within the cell. Accordingly, numerous kinases have been shown to phosphorylate hDlg [4,13,14]. We have shown that in response to cell stress, hDlg is hyperphosphorylated by p38γ [15,16]. hDlg is targeted to the cytoskeleton by its association with guanylate kinase-associated protein (GKAP), and p38γ-catalysed phosphorylation of hDlg triggers its dissociation from GKAP, releasing it from the cytoskeleton [15]. To date, PDZ-binding kinase (PBK) [17] and CDK1/2 [18] are the only kinases that regulate the cell cycle and are also linked to phosphorylation of hDlg. In addition, hDlg is phosphorylated during mitosis in HaCaT and HeLa cells but the molecular mechanisms by which hDlg is regulated during mitosis remain obscure [14,17]. Here we addressed this question. Our data confirm that hDlg is phosphorylated in a cell

Abbreviations: CDK, cyclin-dependent kinase; ERK, extracellular-signal-regulated kinase; FACS, flow cytometry analysis; hDlg, human disc-large; HPV, human papillomavirus; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast.

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cycle-dependent manner, with maximal phosphorylation at mitosis. We identified three residues phosphorylated in mitosis, some are previously unreported, and were located in the hDlg N-terminal half. Our data also show that hDlg phosphorylation in mitosis is regulated by ERK5 pathway and not by p38γ.

2. Materials and methods

2.1. Reagents

Nocodazole was purchased from Sigma. SB203580 and SP600125 were from Calbiochem, and PD184352 and BIRB0796 were made by custom synthesis [19]. All anti-hDlg antibodies were generated as previously described [15]. Anti-JNK1/2 was from New England Biolabs; anti-ERK5 was from the Division of Signal Transduction Therapy (Dundee, UK). Anti-phospho-Ser10 Histone H3 was from Upstate and anti-Cyclin B1 from BD Pharmingen. Anti-p38γ antibody was raised and purified as described elsewhere [20]. All secondary antibodies were from Molecular Probes (Invitrogen) and Perbio Science UK.

2.2. Cell culture and lysis

Human HeLa cells and mouse embryonic fibroblasts (MEF) were cultured in DMEM with 10% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 100 μM nonessential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were treated with 0.5 M sorbitol for 20 min, or synchronised with either nocodazole or thymidine, then lysed in (50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 50 mM sodium β-glycerophosphate, 5 mM pyrophosphate, 0.27 M sucrose, 0.1 mM phenylmethylsulphonyl fluoride, 1% Triton X-100) plus 0.1% 2-mercaptoethanol, 1 mM benzamidine and 0.1 mM PMSF. Lysates were centrifuged (13 000*g*, 15 min, 4 °C), supernatants removed, snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Immunofluorescence

HeLa cells cultured in coverglass were fixed in 4% paraformaldehyde (10 min, room temperature (RT)), blocked in 5% bovine serum albumin in phosphate-buffered saline (PBS) (30 min, RT), then incubated with the appropriate primary antibody (10 μg/ml anti-hDlg, 12 μg/ml anti-hDlg(pS158) and 1:1000 anti-phosho-H3) and secondary antibody. Coverslip were mounted in 50% glycerol in PBS containing DAPI. Cells were analysed by Epifluorescence microscopy (Olympus).

2.4. Cell cycle synchronisation

HeLa cells were arrested in G2/M by adding 50 ng/ml nocodazole (12 h). Mitotic cells were dislodged by gently tapping the plates and then placed in sterile tubes for lysis and analysis. Cells were synchronised at the G1/S phase using a standard double-thymidine block-and-release protocol. Briefly, cells were incubated with 2 mM thymidine (16 h), washed twice with PBS, incubated for 8 h in complete medium, followed by a second 14 h thymidine block. Cells were then released into complete medium for the times indicated. Cell cycle arrest was confirmed by FACS analysis.

2.5. Flow cytometry analysis (FACS)

Cells were trypsinised, resuspended in ice-cold 70% ethanol, and fixed (4 °C, 30 min). Cells were then resuspended in PBS, centrifuged (1000*g*, 4 °C, 10 min), resuspended in 1 ml Beckman Coulter DNAprep and incubated (37 °C, 30 min). Cells were then analysed on a Cytomics FC500 cytometer (Beckman-Coulter).

2.6. Western blotting

Protein samples were subjected to SDS–PAGE and transferred to nitrocellulose. Membranes were blocked in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.5% Tween (TBST) containing 10% skimmed milk powder; and then incubated in TBST/10% skimmed milk powder, and 0.5–1 μg/ml antibody (2 h, RT or overnight at 4 °C), followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualised with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

2.7. Identification of GST-hDlg phosphorylation sites

HeLa cells were transfected with wild type GST-hDlg (36 h) as described in [21]. Cells were then untreated (asynchronised) or treated with nocodazole for 12 h (synchronised in mitosis) and lysed. Lysates clarified by centrifugation (26 000*g*, 4 °C, 15 min). GST-hDlg was partially purified on GSH-Sepharose (GE Healthcare) and washed three times with 1 ml lysis buffer containing 0.5 M NaCl and twice with 1 ml lysis buffer. Samples were treated as described elsewhere [22]. To identify phosphorylation sites, tryptic digests were analysed by liquid chromatography–mass spectrometry on an Applied Biosystems 4000 QTRAP system with precursor ion scanning [23]. The resulting MS/MS data were searched using the Mascot search algorithm (http://www.matrixscience.com) on a local server.

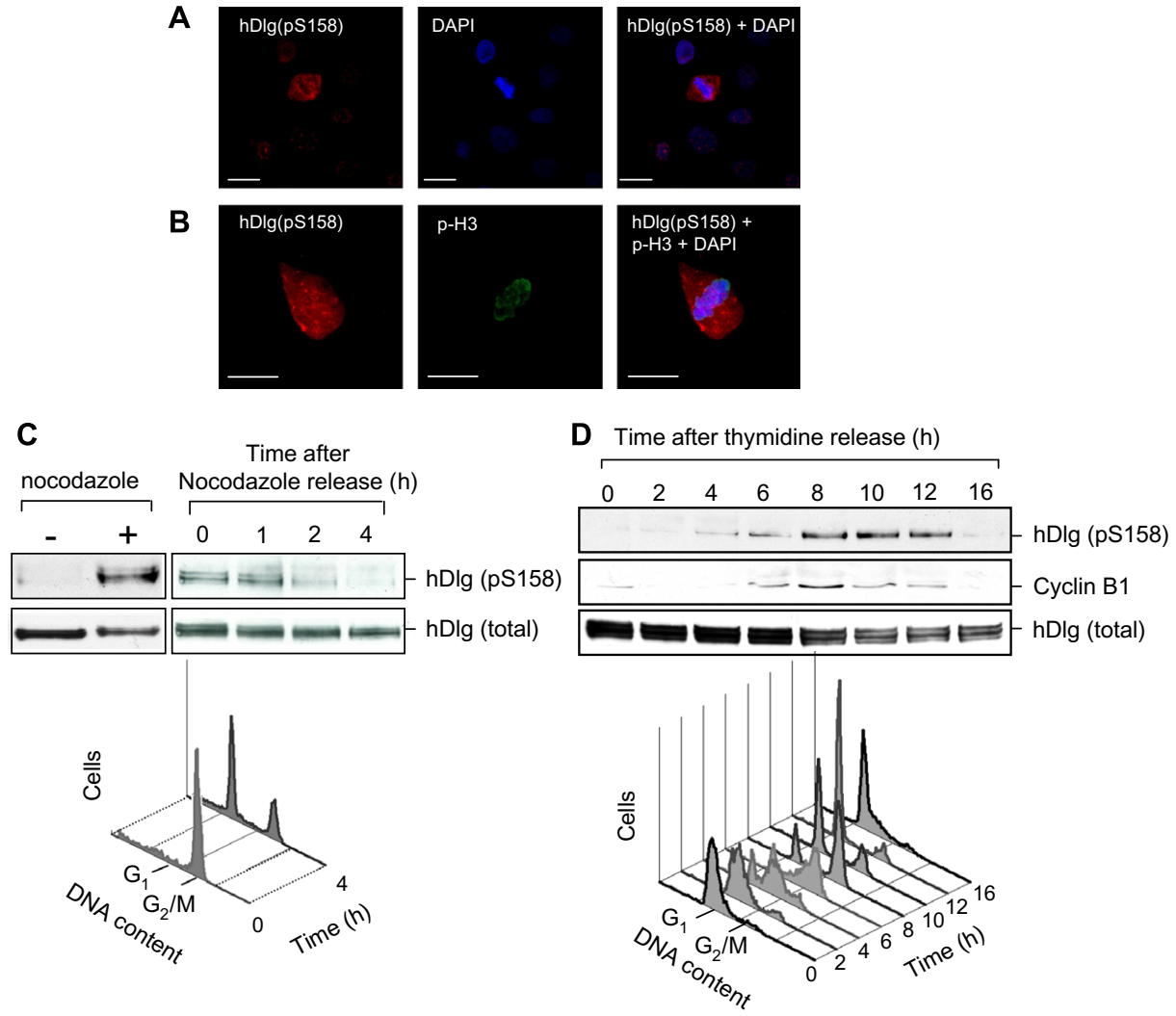
3. Results and discussion

3.1. hDlg is phosphorylated during mitosis

We have previously reported that, when cells are exposed to stress, endogenous hDlg is phosphorylated in the residue Ser158 by the kinase p38γ. Therefore we initiated experiments to examine hDlg phosphorylation during mitosis, using hDlg(pS158), an antibody that specifically recognises hDlg phospho-Ser158 [15]. We first analysed by microscopy hDlg phosphorylation in resting asynchronous HeLa cell and found that hDlg(pS158) stained cells that exhibit condense chromatin typical from mitotic cells (Fig. 1A). To verify that hDlg was phosphorylated in cells in mitosis, cells were fixed and co-stained with anti-hDlg(pS158) and -phosphohistone H3, a mitotic marker. We found that both antibodies stained the same cells demonstrating that hDlg is phosphorylated at Ser158 in mitosis (Fig. 1B).

To further confirm hDlg phosphorylation during mitosis (M phase), we treated HeLa cells with the microtubule-destabilizing agent nocodazole for 12 h to arrest cells in mitosis. Endogenous hDlg from total cell extracts was visualised by Western blot using the anti-hDlg(pS158) and also anti-hDlg antibody, which recognises both phosphorylated and dephosphorylated forms of the protein [15,21] (Fig. 1C). Nocodazole treatment led to accumulation of 90% of the cells in mitosis, as well as to phosphorylation of hDlg at Ser158 (Fig. 1C). hDlg phosphorylation decreased when cells were released from nocodazole treatment and entered again in the cell cycle (Fig. 1C). In addition, HeLa cells were synchronised in G_1/S by double-thymidine block, then released into fresh medium and cell extracts prepared at different time points. FACS analysis showed that cells cycled synchronously between S, M and G_1 phase (Fig. 1D, bottom). The mitotic window was between 6 and 12 h post-release, as shown by cyclin B1 expression and phosphorylated hDlg was detected only during mitosis (Fig. 1D).

To explore the possibility that hDlg could be phosphorylated in unknown residue(s) during mitosis, we used phosphopeptide



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	number	Phosphorylated peptide sequences	Phosphorylated residues
Asynchronised cells	1	EQMMNSSVSS*GS*GSLR	Ser 538* or Ser 540*
	2	GDKG**pS**FNDKR	**Ser 637**
	3	DQSEQETSDADQHVTS*NAS*DS*ESSYR	Ser 669* or Ser 672* or Ser 674*
Cells synchronised in Mitosis	4	QCEPVQPGNPWESGSLSSAAVTSESLP GGGL**pS**PPVEK	**Ser 102**
	5	l**pS**PQVPNEVLGPELVHVSEK	**Ser 122**
	6	SLSEIENVHGFVS*HS*HIS*PIK	Ser 154* or Ser 156* or **Ser 158***
	7	EQMMNSSVSS*GS*GSLR	Ser 538* or Ser 540*
	8	GDKG**pS**FNDKR	**Ser 637**
	9	DQSEQETSDADQHVTS*NAS*DS*ESSYR	Ser 669* or Ser 672* or Ser 674*

Fig. 1. hDlg is phosphorylated in mitosis. Asynchronous HeLa cells were stained with (A) anti-hDlg(p158), or with (B) both anti-hDlg(p158) and anti-pH3 antibodies. Stained cells were analysed by fluorescence microscopy. DNA was DAPI-stained. (C) HeLa cells exposed to nocodazole (12 h) and then released into fresh medium for the times indicated. Top panel; endogenous hDlg was immunoprecipitated from 0.4 mg lysate, using 2 mg of anti-hDlg antibody coupled to protein G-Sepharose. Pellets were immunoblotted with anti-hDlg(pS158) or anti-hDlg antibody. Bottom panel; cells were collected at times indicated and the DNA content analysed by FACS. (D) Cells were released from a double-thymidine block into fresh medium. Top panel; hDlg was immunoprecipitated as in (C) at the times indicated. Extracts (10 μg) were also immunoblotted with anti-cyclin B1 antibody. Bottom panel; cells were collected at times indicated as in (C). Similar results were obtained in three experiments. (E) GST-hDlg expressed in HeLa cells and partially purified from cells untreated or treated with nocodazole. Samples were processed as described (see Section 2). The phosphorylated peaks identified are listed in the table, with more certain phosphorylated residues identified in bold (pS) and residues with less certain assignments marked with an asterisk (S*).

mapping to identify phosphorylation sites. GST-hDlg, expressed in asynchronised HeLa cells or synchronised in mitosis (Fig. 1E), was digested and the resulting phosphopeptides analysed by liquid chromatography–mass spectrometry. The analysis revealed that hDlg was phosphorylated on three peptides containing the following possible phosphorylated residues: Ser637 (peptide 1), Ser538

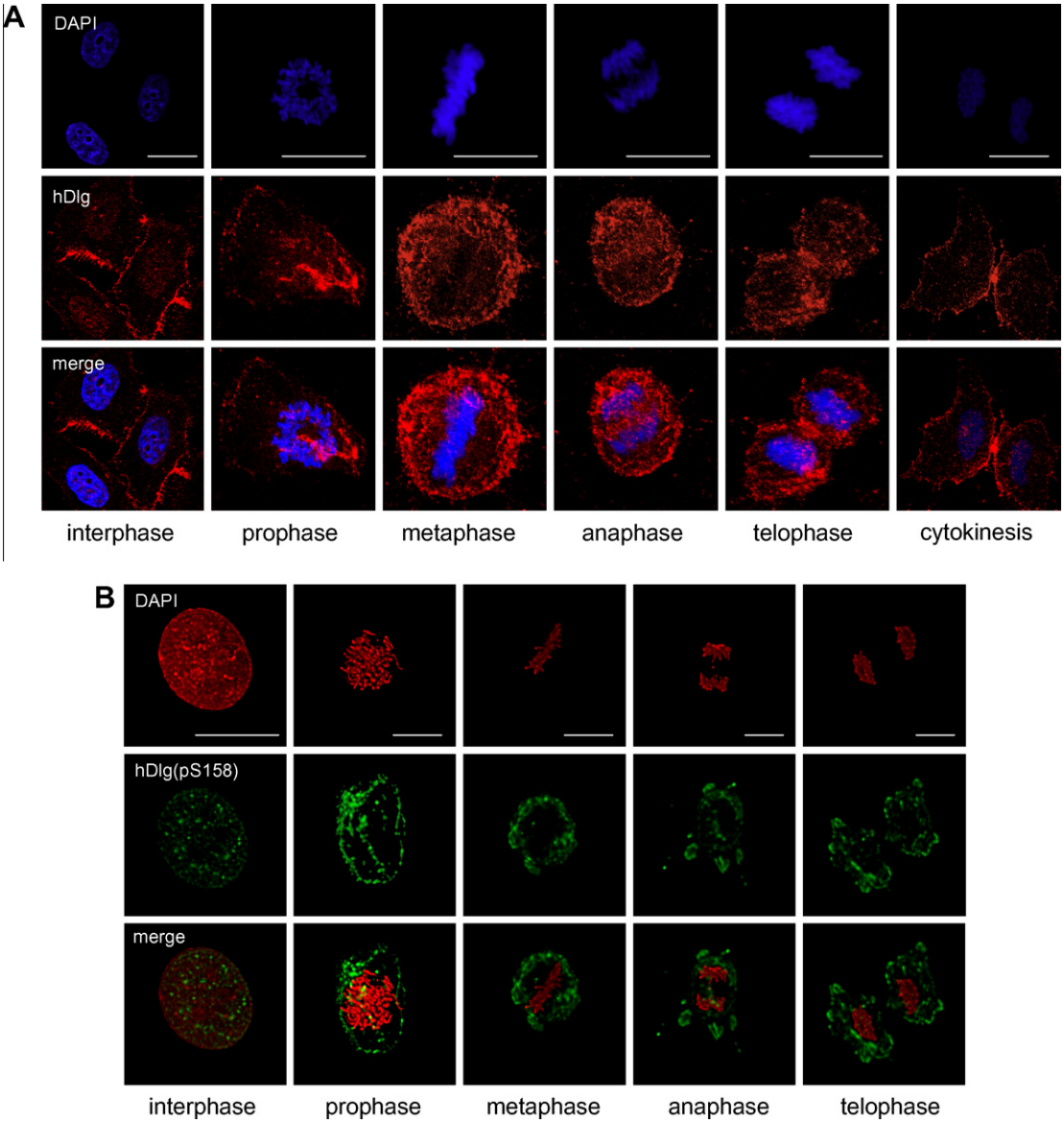

Fig. 2. Localisation of phosphorylated hDlg. Asynchronous HeLa cells were stained with (A) anti-hDlg, or with (B) anti-hDlg(p158) antibody and analysed by fluorescence microscopy. DNA was DAPI-stained. Images shown in (B) were analysed with the program Imaris^{R} 3D reconstruction. Similar results were obtained in four experiments.

or Ser540 (peptide 2), and Ser669/Ser672 or Ser674 (peptide 3) in asynchronised cells, and on at least three additional sites, Ser102, Ser122 and Ser158 (peptides 4 to 6) in mitotic cells. Using mass spectrometric analysis, we were unable to establish the identity of the residue phosphorylated on hDlg tryptic peptide 6, containing Ser154, Ser156 and Ser158. However, the use of anti-hDlg(pS158) indicated that Ser158 was the residue phosphorylated in this peptide (Fig. 1E). Phosphorylation of hDlg tryptic peptides (peptides 1 and 3) containing Ser538 and Ser540, and Ser669, Ser672 and Ser674 were also observed in mitotic cells, but we were unable to establish the precise residue phosphorylated in each peptide (Fig. 1E). These results show that hDlg is phosphorylated at multiple sites during mitosis, indicating phosphorylation at Ser102, Ser122 and Ser158. We nonetheless consider it likely that there are other additional phosphorylation sites. The generation of phospho-specific antibodies that recognise the sites identified here will be needed to determine how their phosphorylation is timely regulated during cell cycle progression and by which kinase(s).

3.2. hDlg distribution during mitosis

Whereas cellular localisation of hDlg has been shown [24,25], the analyses of the pattern distribution for phospho-hDlg during mitosis have not been reported. To investigate this further, we compared the pattern of both hDlg and phosphorylated hDlg expression during the different stages of mitosis in HeLa cell. Total hDlg and phospho-hDlg were detected in asynchronous cells, using anti-hDlg and hDlg(pS158) antibodies, respectively. The cells were also counterstained with DAPI in order to detect the chromosome pattern. As previously described, in normal growing cells that are not progressing through mitosis (interphase), hDlg was largely localised at the cell periphery at sites of cell–cell contact and also in the nuclei with very little expression in the cytoplasm [21,24]. As the cells enter prophase, and progress through metaphase and anaphase, there was a marked accumulation of hDlg in all cellular compartments (Fig. 2A). However, as cells enter telophase and begin cytokinesis, there was a clear concentration of hDlg at the contact point between the two daughter cells, at the midbody (Fig. 2B).

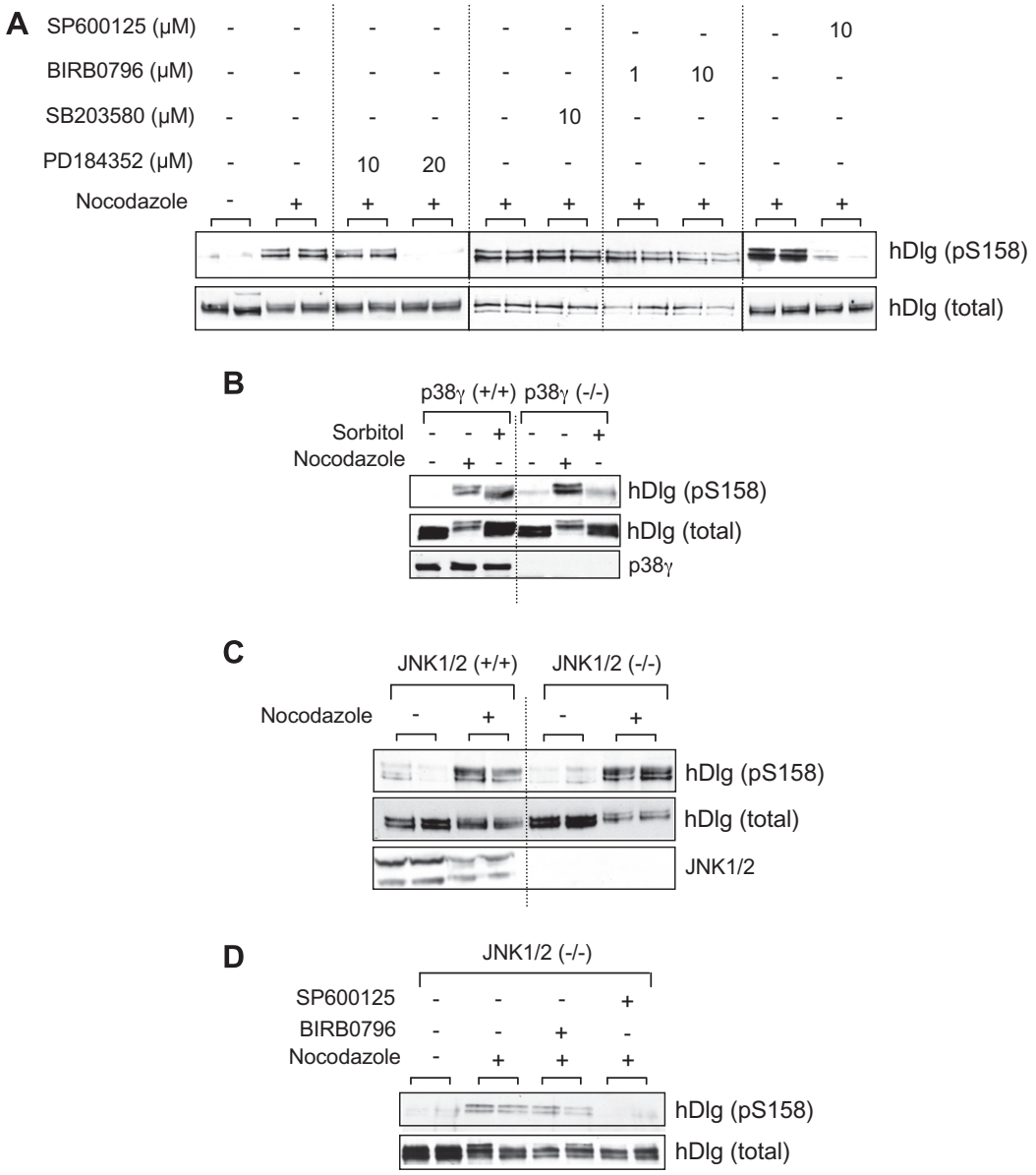

Fig. 3. Phosphorylation of hDlg in mitosis is not mediated by either p38γ or JNK1/2. (A) HeLa cells exposed to nocodazole (12 h) in the presence or absence of different kinase inhibitors, at the concentrations indicated in the figure. Endogenous hDlg was immunoprecipitated from cell lysate, as indicated in Fig. 1 and pellets immunoblotted with anti-hDlg(pS158) or with anti-hDlg antibody. (B) Wild type (p38γ$^{+/+}$) or p38γ-deficient (p38γ$^{-/-}$) MEF were treated with sorbitol or with nocodazole, and the phosphorylation of endogenous hDlg analysed as in (A). p38γ levels were used as loading control. (C) Wild type (JNK1/2$^{+/+}$) or JNK1/2-deficient (JNK1/2$^{-/-}$) MEF were treated with nocodazole, and the phosphorylation of endogenous hDlg analysed as in (A). JNK1/2 levels were used as loading control. (D) JNK1/2$^{-/-}$ MEF were treated with nocodazole as in (A), in the presence or absence of either 10 μM BIRB0796 or 10 μM SP600125. Phosphorylation of endogenous hDlg analysed as in (A). Blots are representative of three independent experiments.

These results are in agreement with the previously described by other laboratories [24,25].

We next investigated phospho-hDlg localisation. Fig. 2B shows that in interphase cells, the localisation of phosphorylated hDlg is restricted to the nucleus in structures that resembled nuclear speckles. Moreover, the progression through mitosis led to an apparent increased level of phosphorylated hDlg (Fig. 2B). When cells entered prophase and then in metaphase and anaphase, there was a marked accumulation of phospho-hDlg at the cell limits and in the cytoplasm (Fig. 2B). In telophase, however, phospho-hDlg was everywhere in the cell and excluded from the contact point between the two daughter cells, contrary to the observed for total hDlg localisation (Fig. 2A). The midbody is the last remnant of the mitotic spindle and is composed of tight bundles of antiparallel microtubules as well as numerous nuclear matrix proteins [26]. Defects in its formation result in failures of cell separation. It has been suggested that hDlg plays a functional role in the maintenance of midbody architecture during cytokinesis [25]. The absence of phospho-hDlg in the midbody suggests that phosphorylation causes hDlg dissociation from this cellular structure. Accordingly, we have previously shown that hDlg phosphorylation affects its protein-binding pattern [15]. Phosphorylation might change not only hDlg localisation but also the binding to different protein complex facilitating its biological function in mitosis. Our results also indicate that only a fraction of the total pool of hDlg protein in the cell is phosphorylated. Since multiple hDlg isoforms can be produced in the cell due to several short insertion elements that arise from alternative splicing [27], we cannot rule out the possibility that hDlg phosphorylation during cell cycle could be splice variant dependent. Thus it has been shown that different hDlg variants un-

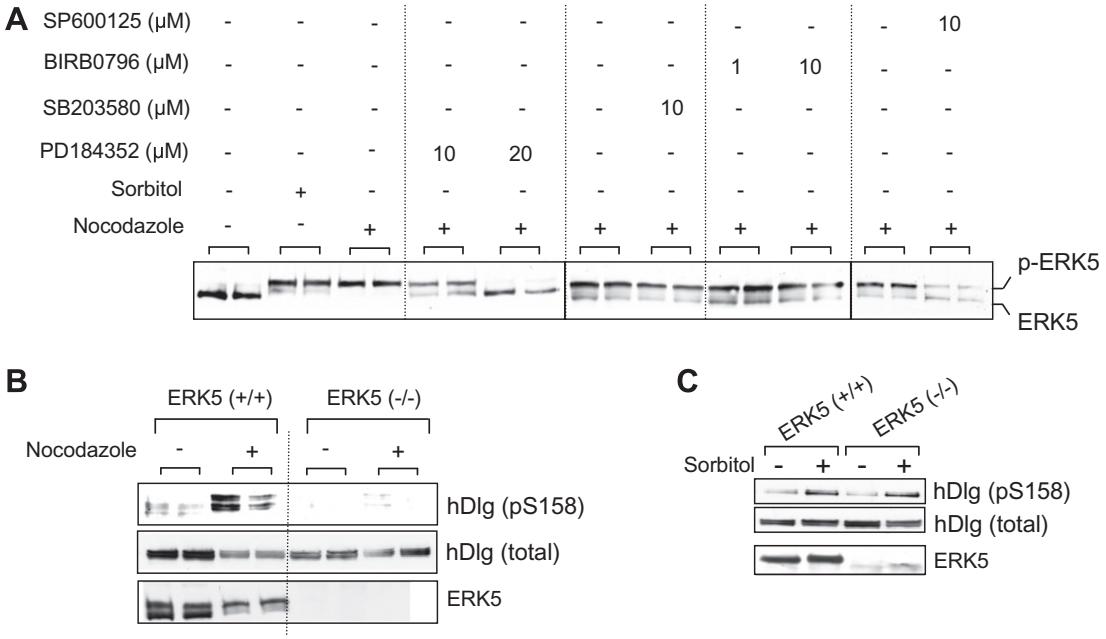

Fig. 4. hDlg is not phosphorylated in ERK5-deficient cells during mitosis. (A) HeLa cells exposed to nocodazole (12 h) in the presence or absence of different kinase inhibitors, at the concentrations indicated in the Figure. Endogenous ERK5 was analysed from 30 μg extract, by immunoblot with anti-ERK5 antibody. (B) Wild type ($ERK5^{+/+}$) or ERK5-deficient ($ERK5^{-/-}$) MEF were treated with nocodazole as in (A), and the phosphorylation of endogenous hDlg analysed as in Fig. 1C. ERK5 levels were used as loading control. (D) $ERK5^{+/+}$ and $ERK5^{-/-}$ MEF were treated with sorbitol as in Fig. 3B and phosphorylation of endogenous hDlg analysed. Blots are representative of three independent experiments.

der certain conditions might have different effects upon cell proliferation [28].

3.3. hDlg is not phosphorylated by p38γ or JNK1/2 during mitosis

To determine which kinase was responsible for hDlg phosphorylation during cell cycle, we arrested the cells in mitosis in the presence or absence of different MAPK pathway inhibitors, and then checked hDlg phosphorylation at the residue Ser158 using anti-hDlg(pS158). Both p38MAPK inhibitors, BIRB0796, which at high concentrations inhibits all p38 and JNK isoforms, and SB203580, which inhibits the isoforms p38α/β in response to osmotic shock ([19] and data not shown), failed to abolish hDlg phosphorylation in mitosis (Fig. 3A). These data indicate that p38γ did not regulate hDlg phosphorylation in mitosis. To confirm this, MEFs deficient in p38γ ($p38\gamma^{-/-}$) were arrested in mitosis and the phosphorylation of hDlg was analysed and compared to that in WT ($p38\gamma^{+/+}$) MEFs. The lack of p38γ did not impaired hDlg phosphorylation during mitosis, although abolished it in response to the osmotic stress sorbitol (Fig. 3B).

Since it has been shown that other MAPKs such as ERK1/2, ERK5 or JNK, are also implicated in the regulation of cell cycle progression, we investigated whether they mediated hDlg phosphorylation during M phase. We treated the cells with PD184352, which at low concentration abolishes ERK1/2 activation and at high concentration the activation of ERK5, and with SP600125, which blocks JNKs activity among other many protein kinases [29]. Both inhibitors blocked hDlg mitotic phosphorylation in cells treated with nocodazole (Fig. 3A). PD184352 blocked hDlg phosphorylation at high concentration suggesting that ERK5, rather than ERK1/2, regulated hDlg phosphorylation in M phase. To verify whether SP600125 inhibited hDlg phosphorylation through JNK1/2 or other kinase, we analysed hDlg phosphorylation in MEF deficient in JNK1/2 ($JNK1/2^{-/-}$) arrested in mitosis. We found that in M phase, hDlg phosphorylation in $JNK1/2^{-/-}$ MEFs was similar to that in $JNK1/2^{+/+}$ MEFs (Fig. 3C). However, when arrested $JNK1/2^{-/-}$ MEFs were also treated with SP600125, but not with BIRB0796, hDlg phosphorylation was impaired. These results together demonstrate that neither p38MAPKs nor JNK1/2 mediate hDlg phosphorylation during mitosis, and suggest that it is controlled by the ERK5 pathway.

3.4. ERK5 pathway mediates hDlg phosphorylation in M phase

Since high concentration of PD184352 blocked hDlg phosphorylation, we next examined ERK5 phosphorylation during mitosis in the present of different kinase inhibitors. ERK5 from cell extracts was visualised by Western blot using an anti-ERK5 antibody that recognises equally both phosphorylated and dephosphorylated forms of the protein. Phosphorylation of ERK5 causes retardation on its electrophoretic mobility. Nocodazole treatment led to ERK5 band-shift comparable to that observed after osmotic shock treatment, which also causes ERK5 phosphorylation (Fig. 4A). ERK5 band-shift was not blocked by BIRB0796 and SB203580, was partially blocked by SP600125, and completely abolished at high concentration of PD184352 (Fig. 4A). These data are in agreement with the results observed in hDlg phosphorylation in mitosis and indicate that ERK5 controls it.

To verify whether ERK5 regulated hDlg phosphorylation, we checked hDlg in MEF deficient in ERK5 ($ERK5^{-/-}$) arrested in mitosis. We found that in M phase, hDlg phosphorylation in $ERK5^{-/-}$ MEFs was significantly diminished compared to that in $ERK5^{+/+}$ MEFs (Fig. 4B). hDlg phosphorylation was not impaired in $ERK5^{-/-}$ MEFs since the treatment with osmotic shock triggered a hDlg phosphorylation similar to WT MEFs (Fig. 4C). These results show that ERK5, either directly or through other kinase, mediates hDlg phosphorylation during mitosis.

In conclusion, using specific kinase inhibitors as well as cells deficient in different kinases, we report for the first time that hDlg phosphorylation in M phase might be regulated by the ERK5 pathway. ERK5 phosphorylation during mitosis and its implication in the cell cycle control has been previously described [30,31], although the precise mechanism by which ERK5 functions in this

process is unclear. We suggest that the new signalling pathway leading to ERK5 pathway activation and hDlg hyperphosphorylation must occur for a correct timely mitotic entry and progression through mitosis.

Acknowledgments

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