CDK phosphorylation of the discs large tumour suppressor controls its localisation and stability

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Summary
The Discs Large (Dlg) protein is known to be involved in the regulation of cellular proliferation and polarity in a variety of tissues. The human homologue DLG1 is thought to be a tumour suppressor, through formation of a complex with the APC (adenomatous polyposis coli) protein, causing negative regulation of the cell cycle. An alternative oncogenic role has also been proposed, in which the PI3-kinase pathway is activated under the influence of the adenovirus E4 ORF1 protein. The differing roles seem to be related to differences in the precise pattern of expression. However, the biochemical pathways involved in regulating Dlg1 function during different phases of the cell cycle remain unclear. In this study we show that phosphorylation is a major post-translational modification of the protein and that it affects both location and function. DLG1 lies at the cellular junctions in G1, is enriched in the cytoplasm in S phase and localises to the mitotic spindle in M phase. We also show that DLG1 is phosphorylated by both CDK1 and CDK2 on Ser158 and Ser442. These phosphorylated sites together affect the nuclear localisation of the protein, and implicate the role of phosphorylation on Ser158 and Ser442 in its putative nuclear functions as a tumour suppressor. In addition, the mutants at these sites demonstrate different half-lives as well as different susceptibilities to ubiquitination, suggesting a role for these phosphorylation events in controlling DLG1 protein stability. These findings establish phosphorylation events as key regulators of DLG1 localisation and function.

Key words: Dlg (Discs Large), Phosphorylation, Cell cycle

Introduction
The discs large homologue 1 (DLG1) protein, also known as hDlg or SAP-97, is a representative member of a growing family of proteins collectively termed membrane-associated guanylate kinase homologues (MAGUKs), which include the ZO (zonula occludens) proteins ZO1, ZO2 and ZO3 and CASK (calcium/calmodulin-dependent serine kinase protein). It is the mammalian homologue of Drosophila discs large (Dlg), and has multiple protein domains including three PDZ motifs, an L27 domain, an SH3 domain, and a guanylate-kinase-homology (GUK) domain (Hough et al., 1997; Kim, 1997; Craven and Breitd, 1998; Gonzalez-Mariscal et al., 2000).

Drosophila Dlg has long been known to be involved in cell growth control, maintenance of cell adhesion and cell polarity (Bilder et al., 2000; Caruana, 2002) in both embryonic and adult tissues and its inactivation results in the neoplastic growth of imaginal disc epithelial cells (Bryant and Schmidt, 1990; Woods and Bryant, 1991; Woods and Bryant, 1993; Woods et al., 1996; Goode and Perrimon, 1997). Transgenic expression of human DLG1 in Drosophila harbouring Dlg mutations reverses the neoplastic phenotype (Thomas et al., 1997), showing retention of the Dlg tumour suppressor function between humans and flies. Specific PDZ domains of DLG1 have been shown to interact with the C-termini of several proteins, including the mitotic kinase TOPK/PBK (Gaudet et al., 2000), the human papillomavirus (HPV) E6 oncoprotein (Kiyono et al., 1997; Lee et al., 1997), the HTLV-1 Tax oncoprotein (Suzuki et al., 1999), the adeno-viral E4 ORF1 (Lee et al., 1997) and the APC tumour suppressor protein (Matsumune et al., 1996). Since DLG1 forms a complex with APC, it is thought that it will function in processes that regulate cell polarity and proliferation in response to cell contact in epithelial cells. A recent study has shown that Cdc42 and the Par6-PKCζ complex regulate the spatial association of DLG1 and APC, and this physical interaction between them is necessary for the polarisation of the microtubule cytoskeleton (Etienne-Manneville et al., 2005). Interestingly, the interaction of HPV E6, adenoviral E4 ORF1 and HTLV-1 Tax all interfere with the interaction between DLG1 and APC, and thereby perturb normal cell growth control. In the case of HPV E6, this interaction is unique to those HPV types that are associated with the development of malignancy: so-called low risk or benign HPV E6 proteins do not possess the PDZ-binding motifs and do not interact with DLG1. Moreover, HPV E6 can target DLG1 for ubiquitin-mediated degradation (Gardioli et al., 1999; Pim et al., 2000; Kühne et al., 2000), probably by enhancing a normally occurring process, because DLG1 appears to be ubiquitinated in cells even in the absence of E6 (Gardioli et al., 1999). The oncogenic potential of the adenovirus E4 ORF1 protein also correlates with its binding to Dlg1 and other cellular PDZ proteins such as MUPP1, MAG1 and ZO2 via a C-terminal PDZ-binding motif. However, recent studies have shown that, upon binding to the adenovirus E4 ORF1, DLG1 containing the I3 isoform might also have oncogenic potential by activating the phosphoinositide 3-kinase (PI3K) pathway (Frese et al., 2006).

Numerous kinases have been shown to participate in the phosphorylation of DLG1 and to induce functional and positional changes in it. These include the p56lk tyrosine kinase which targets the N-terminus of DLG1 and causes coupling of tyrosine kinase and the voltage-gated potassium channel in T cells (Hanada et al., 1997), CaMKII kinase, which regulates Dlg1 localisation at the synapse (Koh et al., 1999), Jun N-terminal kinase (JNK), which phosphorylates DLG1 in response to osmotic stress and leads to its accumulation at cell-cell contacts (Massimi et al., 2006), the PAR-
1 kinase, which controls postsynaptic targeting of Dlg1 to neuromuscular junctions (Zhang et al., 2007) and the p38 MAPK, which dissociates DLG1 from GKAP and consequently triggers its release from the cytoskeleton (Sabio et al., 2005). Hyperphosphorylation of DLG1 has been shown to lead to its interaction with the β-TrCP ubiquitin ligase receptor leading to ubiquitylation of the protein and a decrease in its stability (Mantovani and Banks, 2003). The PDZ-binding kinase (PBK) (Gaudet et al., 2000) is the only kinase that has so far been linked to phosphorylation of DLG1, which regulates the cell cycle. However there is no direct evidence that DLG1 is a substrate for regulatory cell cycle kinases, nor is there any information on probable biological consequences of its phosphorylation during the cell cycle.

Here we report the phosphorylation-dependent regulation of DLG1 by two principal cell cycle regulatory kinases, the cyclin-dependent kinases 1 and 2 (CDK1 and CDK2). Both phosphorylation events take place on two residues within the N-terminal half of the protein. Most strikingly, mutants of DLG1 that cannot be phosphorylated or that mimic phosphorylation on those sites regulate the localisation and the stability of the protein. These studies directly link DLG1 function to the control by the cell cycle and highlight the general importance of phosphorylation in the function of the protein.

**Results**

**Cell cycle regulation of DLG1**

Many kinases have been implicated in the regulation of the cell cycle, including the CDKs, the polo-like kinases (PLKs) and the mitogen-activated protein kinase (MAPKs) to name a few. They function by influencing the regulation of the function of their various substrates (Bregman et al., 2000; Geisen and Moroy, 2002; Sherr and McCormick, 2002). Previous studies have also shown that DLG1 can act negatively to suppress G0-G1 to S phase progression (Ishidate et al., 2000; Matsumine et al., 1996), and that it is hyperphosphorylated during M phase (Gaudet et al., 2000).

In addition, there are several reports detailing different patterns of expression of DLG1, including the presence of cytoplasmic, cytoskeletal-bound and nuclear forms of the protein (Roberts et al., 2007; Cavatorta et al., 2004), some of which are indeed regulated by differential phosphorylation events (Massimi et al., 2004; Sabio et al., 2006). However, no detailed studies have been performed to attempt to link these apparently diverse aspects of DLG1 localisation and function with the cell cycle. Therefore we performed a series of studies to investigate the pattern of DLG1 expression during the cell cycle. To do this, we first analysed the pattern of DLG1 expression in asynchronously growing HaCaT skin epithelial cells. As can be seen from Fig. 1A, DLG1 expression patterns were diverse, with cytoplasmic, diffuse and membrane-bound forms, as well as structures, such as the midbody and the mitotic spindle clearly visible, suggesting potentially different patterns of expression, depending upon the phase of the cell cycle. The specificity of the assay was verified using HaCaT cells stably expressing an shRNA against DLG1 (Massimi et al., 2008). As can be seen from Fig. 1B these cells are devoid of detectable DLG1 protein and the anti-DLG1 antibody used shows no signal in these cells, confirming that the different patterns of expression observed in Fig. 1A is indeed due to specific changes in the pattern of DLG1 expression. To investigate this further we then proceeded to analyse the pattern of DLG1 expression in HaCaT cells that had been synchronised following a block with aphidicolin. Cells were released and fixed at different times and then analysed for the pattern of DLG1 expression by immunofluorescence. The results obtained are shown in Fig. 1C, together with the corresponding FACS analysis in Fig. 1D. In the G1 population, DLG1 is highly membrane bound. However its localisation progressively becomes much more cytoplasmic as the cells progress through the cell cycle, with an overall diffuse staining when the cells become more rounded as they approach M phase. The protein shows a marked relocation to the mitotic spindle at mitosis and it finally accumulates strongly at the midbody at cytokinesis, in agreement with previous studies (Massimi et al., 2003). Taken together, these data suggest that DLG1 is relocalised to different cellular compartments during different phases of the cell cycle.

**Dlg is phosphorylated by CDK1 and CDK2**

The only reported kinase potentially involved in the M phase phosphorylation of DLG1 is the PDZ binding kinase (PGBK) (Gaudet et al., 2000). Since we found that DLG1 is subject to differential patterns of expression during the cell cycle, we hypothesised that other kinases may be involved, two obvious candidates being CDK1 and CDK2. To investigate whether DLG1 could be a substrate for these kinases, we performed in vitro phosphorylation assays with purified enzymes and purified GST-DLG1 fusion protein. As Fig. 2B shows, DLG1 is phosphorylated in vitro by both CDK1 and CDK2, although it is generally a better substrate for CDK2 than for CDK1.

Our next aim was to map the region(s) and possibly the residue(s) that are phosphorylated by the two kinases. To do this, we used constructs coding for various deletion mutants of GST-tagged Dlg (Gardiol et al., 2002). The mutants used spanned the entire sequence of the protein separately: the N-terminus, the three PDZ domains and the C-terminal region, including the SH3 and GUK domains, as shown in Fig. 2A. The in vitro kinase assays were carried out with purified CDK1 and CDK2 (Fig 2C,D, respectively), which map the major sites of phosphorylation to residues within the N-terminus and the PDZ region of DLG1. No detectable, or at best marginal, phosphorylation of the C-terminal fragment was obtained with CDK1 and CDK2, respectively.

The DLG1 sequence was then scanned for probable CDK consensus sites (S/T-P-x-R/K, where x represents any amino acid) using ScanSite software (http://scansite.mit.edu/), and two perfect consensus sites were found containing serine residues at 158, which lies in the N terminus, and at residue 442, which lies between PDZ domains 2 and 3. Upon analysis of the human, mouse, rat, *Drosophila* and *Caenorhabditis elegans* DLG1 sequences (Table 1), it was found that though these serines and the corresponding consensus sites are perfectly conserved among the mammals and vertebrates, they were not conserved in *Drosophila* and *C. elegans*. This suggests that these potential phosphorylation sites have roles in DLG1 function in more developed species, but that different pathways regulate Dlg1 in lower organisms.

To verify that these two potential sites are indeed phosphorylated by CDK1 and CDK2, we mutated Ser158 and Ser442 both separately and together, to alanines, to block phosphorylation on the residues. The mutants expressed as GST fusion proteins were then subjected to in vitro phosphorylation assays with purified CDK1 and CDK2. The results obtained are shown in Fig 3A,B. When either residue alone is mutated, DLG1 was still phosphorylated by both kinases with almost wild-type efficiency. Only when both sites were mutated was phosphorylation by CDK1 and CDK2 abolished. This suggests that both serines are equally recognised by the CDKs as being phospho-acceptor sites.
Discs large phosphorylation

DLG1 is found in CDK-containing complexes in vivo

To investigate whether DLG1 could complex with and be phosphorylated by CDKs in vivo, we first performed a series of co-immunoprecipitation experiments in HEK293 cells, immunoprecipitating endogenous CDK2 and then probing for endogenous DLG1. Fig. 4 demonstrates that DLG1 coimmunoprecipitates strongly with CDK2 as well as the CDK1 cyclin subunit, cyclin B, although we were unable to detect an interaction by coimmunoprecipitation of DLG1 and CDK1. This might be a reflection of the relative differences in the efficiency by which CDK1 and CDK2 can phosphorylate DLG1 in vitro, with CDK2 phosphorylation of DLG1 generally being much stronger. Therefore to ascertain whether the M-phase phosphorylation of DLG1 was in part mediated by CDK1, a different approach was taken. The cells were treated for 18 hours with the microtubule-depolymerising agent nocodazole to block cells in mitosis, which was confirmed by FACS analysis (Fig. 4D). The cells were then treated with the CDK1-specific inhibitor roscovitine for 3 hours and DLG1 production was analysed by western blotting. The results obtained are shown in Fig. 4C. A number of slower migrating forms of DLG1 were present in nocodazole-arrested M-phase cells, indicating that multiple phosphorylation events take place on DLG1 in the presence of nocodazole. Intriguingly, the slowest migrating form disappears following treatment with roscovitine, demonstrating that at least one of the M-phase phosphorylation events on DLG1 is mediated by CDK1. For comparison, asynchronously growing cells were likewise treated with roscovitine and the effects on DLG1 production analysed by western blotting. As can be seen from Fig. 4C there was also a marked decrease in the intensity of the slower migrating phosphorylated form of DLG1 (Massimi et al., 2006), further supporting the notion that DLG1 is a substrate for CDKs in vivo.

Phosphorylation on Ser158 and Ser442 enhances nuclear expression of DLG1

To investigate the role of the CDK phosphorylation events on DLG1 function, we first analysed the effects of mutating these residues upon the pattern of DLG1 expression. To do this, a series of HA-tagged mutants were generated: Ser158→Ala and Ser158→Asp, Ser442→Ala and Ser442→Asp, Ser158Ser442→AlaAla and Ser158Ser442→AspAsp, where mutations to alanine block phosphorylation, and those to aspartic acid mimic phosphorylation. To verify that the different DLG1 mutants were expressed, HEK293 cells were transfected with the different expression plasmids, and after 24 hours, protein levels were ascertained by western blot analysis. The results obtained are shown in Fig. 5A. Interestingly, wild-type DLG1 migrated as two distinct forms on the gel, the slower of which is more heavily phosphorylated (Caruana and Bernstein, 2001; Massimi et al., 2006), whereas the double Ala mutant co-migrated with the faster migrating band, and the double Asp mutant with the
slower migrating phosphorylated form of DLG1. In addition, the single point mutations at Ser158 had essentially a similar phenotype, demonstrating that the presence or absence of a single acidic residue at this position has a profound effect on the overall conformational structure of DLG1, and that a single acidic residue at position 158 is sufficient to account for the slower migrating form of DLG1. A similar pattern of migration was also obtained when the mutants were expressed in U2OS cells (data not shown).

We then proceeded to investigate the effects of the two double point mutations upon the pattern of DLG1 expression in vivo. U2OS cells were transfected with the different expression plasmids and DLG1 expression ascertained by immunofluorescence using anti-HA antibodies. The results obtained in Fig. 5B show the predominant patterns of expression that were observed. Wild-type DLG1 exhibited two distinct patterns of expression: a diffuse staining of the entire cell, and a staining that excluded the nucleus. However, the point mutant Ser158Ser442→AlaAla showed a predominantly nuclear excluded pattern, whereas the Ser158Ser442→AspAsp had a more diffuse pattern of staining. To accurately quantify this, 300 cells were counted for each assay and the expression scored as diffuse or nuclear exclusion (Fig. 5C), which confirmed the differential pattern of expression of the two mutant proteins.

To further demonstrate a direct link between CDK activity and the pattern of DLG1 localisation, we analysed the pattern of DLG1 expression in HaCaT cells in the presence and absence of the CDK inhibitor roscovitine (Fig. 5D). Treatment with roscovitine induced an overall decrease in the intensity of DLG1 staining and a more diffused cytoplasmic pattern of expression. However, there was no apparent change in the ability of DLG1 to localise to the midbody in cytokinesis, although we were unable to detect localisation to the mitotic spindle in the few dividing cells that could be detected. These results demonstrate that inhibition of CDK activity has a direct effect upon the pattern of DLG1 expression.

DLG1 protein stability and susceptibility to ubiquitylation is in part determined by its phosphorylation on Ser158 and Ser442. The above results demonstrate that phosphorylation of DLG1 on Ser158 and Ser442 can affect its cellular pattern of expression. We also noticed from Fig. 5A subtle differences in the levels of expression of the different mutant forms of DLG1 and, because phosphorylation is cell, and a staining that excluded the nucleus. However, the point mutant Ser158Ser442→AlaAla showed a predominantly nuclear excluded pattern, whereas the Ser158Ser442→AspAsp had a more diffuse pattern of staining. To accurately quantify this, 300 cells were counted for each assay and the expression scored as diffuse or nuclear exclusion (Fig. 5C), which confirmed the differential pattern of expression of the two mutant proteins.

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<p>| Table 1. Comparison and sequence alignment of the regions of Dlg containing the consensus CDK phosphorylation sites in human, mouse, rat, Drosophila and C. elegans |</p>
<table>
<thead>
<tr>
<th>Ser158</th>
<th>Ser442</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens DLG1</td>
<td><em>FVSHSHSPIKPTEA</em></td>
</tr>
<tr>
<td>Rattus norvegicus DLG1</td>
<td><em>FVSHSHSPIKPTEA</em></td>
</tr>
<tr>
<td>Mus musculus DLG1</td>
<td><em>FVSHSHSPIKPTEA</em></td>
</tr>
<tr>
<td>Drosophila melanogaster Dlg1</td>
<td><em>LNRQRQTSHDG</em></td>
</tr>
</tbody>
</table>

Sequences were obtained from the ExPASy proteomics server (http://www.expasy.org/). Residues highlighted in red indicate the CDK consensus sequence.
Discs large phosphorylation of proteins has long been connected to their stability in the cell, we asked whether the phosphorylation events on these residues could affect DLG1 protein stability. Cells were transfected with the different expression plasmids, and after 24 hours, further protein synthesis was blocked by treatment with cycloheximide. The levels of DLG1 expression were then analysed over a period of 8 hours by western blotting (Fig. 6A). Strikingly, we found that the DLG1 double Ala mutant has a significantly reduced half-life compared with both wild-type DLG1 and the DLG1 double Asp mutant, with the single point mutants, not surprisingly, giving an intermediate phenotype (data not shown). This suggests that phosphorylation of the protein on these two residues increases its stability and affects the turnover of the protein. To investigate this further, we also performed an in vivo ubiquitylation assay. Cells were transfected with the HA-tagged DLG1 expression plasmids together with a Flag-tagged ubiquitin expression plasmid (Fig. 6B). The inputs for all three proteins were approximately equal; however, following immunoprecipitation of DLG1 and western blot detection for ubiquitin (Fig. 6C) we found that the wild type and the double Ala mutant show a high degree of ubiquitylation. By contrast, the double Asp mutant had only a very low level of ubiquitylation. These results suggest that the acidic status of the Ser158 and Ser442 residues can directly affect the levels to which DLG1 is ubiquitylated, which in turn affects the overall stability of the protein.

DLG1 is phosphorylated in vivo in a cell-cycle-dependent manner on Ser158

The above results provide compelling evidence that DLG1 can be a substrate for CDK phosphorylation on two different residues, and therefore suggests that phosphorylation on these sites will be cell cycle dependent. In order to verify this, we generated rabbit polyclonal phospho-specific antibodies against Ser158-P and Ser442-P. To confirm the specificity of these antibodies in western blotting, we first phosphorylated the GST-DLG1 fusion protein in vitro with purified CDK1 and CDK2 and cold ATP. The proteins were then subjected to gel electrophoresis and western blotting with the phospho-specific antibodies as well as with control anti-DLG1 monoclonal antibody. The anti-DLG1 monoclonal antibody equally recognised both the phosphorylated and non-phosphorylated forms of the protein (Fig 7A,B). By contrast, the phospho-specific antibodies to Ser158 and Ser442 each only recognised the CDK1 and CDK2 phosphorylated forms of GST-DLG1, confirming both the specificity of these antibodies and that the phosphorylation events actually occur on these residues.

We then proceeded to investigate the pattern of reactivity of these phospho-specific antibodies on endogenous DLG1 during different phases of the cell cycle in HaCaT cells. To do this, cells were either grown asynchronously for comparison, or arrested with aphidicolin and subsequently released and analysed throughout the cell cycle. The results demonstrate that the anti-DLG1 monoclonal antibody showed a fairly constant level of reactivity against DLG1 throughout the cell cycle (Fig. 7C) and, although there were slightly lower levels in early G1 phase and an apparent increase during M phase. By contrast, the phospho-specific Ser158 antibodies showed a clear preference for DLG1 reactivity during the S phase and the time points leading to the M phase, compared with the much weaker signals in asynchronously growing cells and in early G1. Similarly, the phospho-specific Ser442 antibodies showed preferential reactivity against DLG1 during S phase.

Finally, we wanted to confirm that the Ser158 phosphorylation event that we detected on DLG1 is indeed a result of CDK activity.
To do this, asynchronously growing HaCaT cells were grown in the absence or presence of roscovitine and DLG1 levels analysed using the phospho-specific antibody directed against Ser158. The results obtained are shown in Fig. 7D, where it can be seen that the ability of the anti-Ser158-P antibody to detect DLG1 was completely lost following treatment with roscovitine. Taken together these results demonstrate that DLG1 is a substrate for CDKs during different phases of the cell cycle, the consequences of which have profound implications for DLG1 localisation and stability.

**Discussion**

In this study, we analysed the regulation of the DLG1 tumour suppressor during the cell cycle. We show that it is a substrate for both CDK1 and CDK2 with concomitant hyper-phosphorylation during the G1-S and M phases. Relocalisation of DLG1 was also observed during different phases of the cell cycle, varying from membrane-bound or diffuse patterns of expression, to an enhanced degree of cytoplasmic expression during S phase, and accumulation on the mitotic spindle during mitosis, as well as in the midbody during cytokinesis. Coupled with a mutational analysis highlighting a role for acidic residues at the CDK phospho-acceptor sites on the pattern of DLG1 localisation and stability, these results demonstrate that CDK phosphorylation of DLG1 on Ser158 and Ser442 directly contributes to its pattern of expression and stability.

The role of Dlg1 has been established as a regulator of cell polarity, adhesion and junction stability (Abbott and Natzle, 1992; Woods et al., 1996; Humbert et al., 2003), as a controller of development (Caruana and Bernstein, 2001; Iizukako et al., 2007) and it has also been shown to have a strong involvement in normalising synapse regulation at the neuromuscular junction (Lahey et al., 1994; Budnik et al., 1996; Zhang et al., 2007). However, the actual role of mammalian DLG1 in the control of cell growth and cell polarity has been a matter of much speculation. This is complicated by the fact that the molecular mechanisms regulating DLG1 function, stability and localisation are still largely unknown. It has certainly been proposed to have a cell cycle regulatory function (Nguyen et al., 2003) through its association with the APC tumour suppressor (Ishidate et al., 2000) and the mitotic PDZ-binding kinase (Gaudet et al., 2000). Recent studies have also suggested that different isoforms under certain circumstances might also have different effects upon cell proliferation through activation of the PI3K pathway (Frese et al., 2003; Frese et al., 2006). This might in fact be linked to reported differences in the cellular localisation of different DLG1 isoforms (McLaughlin et al., 2002; Roberts et al., 2007). It now seems likely that many of these diverse activities and patterns of expression may actually be connected through common pathways of post-translational modification of DLG1. The initial observations supporting this came from studies which showed that DLG1 was phosphorylated via the MAPK pathway following osmotic shock, resulting in altered patterns of protein localisation (Sabio et al., 2005; Massimi et al., 2006). These studies were done under stress conditions, and we now show that similar alterations in the pattern of DLG1 expression are obtained during a normal cell cycle and, most importantly, that this is directly controlled by both CDK1 and CDK2 by phosphorylating DLG1 on two sites, Ser158 and Ser442.

We show that DLG1 is a substrate for CDK1 and CDK2 both in vitro and in vivo. This was done first using purified components in vitro, and mutational analysis allowed us to identify that Ser158 and Ser442 were both phospho-acceptor sites for CDK1 and CDK2.
The data supporting CDK regulation of DLG1 in vivo comes from several experiments. We observe a strong co-immunoprecipitation between endogenous DLG1 and CDK2, as well as between DLG1 and cyclin B, demonstrating that DLG1 can at least be found in the complexes that would be expected to have CDK activity. We also observed a clear difference in the pattern of DLG1 migration in SDS-PAGE, when cells were exposed to the CDK inhibitor, roscovitine, in both G2-M as well as in asynchronously growing cells, suggesting that CDKs do in fact phosphorylate endogenous DLG1. We cannot formally exclude the fact that CDK5 might not be involved, although this seems unlikely because it is predominantly expressed in neural cells. Finally, using phospho-specific antibodies directed against one of these sites, we show a clear cell-cycle-dependent phosphorylation of DLG1 on Ser158 and Ser442, with the reactivity of the antibody against Ser158 being lost after treatment with roscovitine. Interestingly, Ser158 appears to be phosphorylated during late G1 and during M, whereas Ser442 is predominantly phosphorylated during M phase, suggesting that a different hierarchy of phospho-acceptor site recognition on DLG1 might exist during different phases of the cell cycle.

These phosphorylation events appear to have a number of important consequences. One of them seems to be a major structural alteration. DLG1 normally migrates as two major species and interestingly, the Ser158→Asp mutation alone is sufficient to generate the slower migrating form of the protein, suggesting that the presence of an acidic residue at this position induces a major structural change in DLG1. Interestingly, phosphorylation at these sites also appears to regulate the level to which DLG1 is ubiquitylated. Certainly, the lack of acidic residues at Ser158 and Ser442 greatly shortens the half-life of the protein, whereas by contrast, the presence of two acidic residues significantly extends the half-life and decreases the level of ubiquitylation. Obviously, these assays were performed with ectopically expressed mutants and it will now be interesting to determine whether different species of DLG1 have different levels of stability during different phases of the cell cycle.

It is also clear that phosphorylation at Ser158 and Ser442 probably alters the pattern of DLG1 expression, with the double Ala mutant exhibiting a pattern of nuclear exclusion. It is intriguing to note that previous studies also identified Ser158 and Ser442 as being sites of phosphorylation by p38 following exposure of cells to osmotic stress (Sabio et al., 2005), although our studies show that these sites are phosphorylated during the course of a normal cell cycle. As a result of these phosphorylation events following osmotic shock, DLG1 lost its ability to bind GKAP and the cytoskeleton. It is tempting to speculate that phosphorylation of DLG1 during a normal cell cycle by the CDKs at these two sites may also have a similar function and thereby correctly regulate the localisation of DLG1 during different phases of the cell cycle. Studies are now in progress to identify the cellular binding partners of DLG1 whose interactions might be modified as a consequence of these phosphorylation events, with GKAP as the obvious candidate (Wu et al., 2000). It is also significant to note that Ser442 lies between the PDZ2 and PDZ3 domains of the protein, which is an area involved in binding several proteins involved in cell propagation, including tumour suppressors such as the APC (Matsumine et al., 1996), as well as oncoproteins such HPV E6, 9ORF1 and HTLV Tax1 (Kiyono et al., 1997; Mantovani and Banks, 2001; Latorre et al., 2005) and it will be interesting to determine whether there are any cell cycle regulatory aspects to these interactions.

These results provide the first direct evidence of a link between DLG1 and the regulation of the cell cycle through an interaction with the key cell cycle regulators, CDK1 and CDK2, and further highlight the importance of phosphorylation as a major post-translational modification in the life of the protein.
Fig. 7. DLG1 phospho-specific antibodies specifically recognise DLG1 phosphorylated by CDK1 and CDK2. (A,B) GST-DLG1 was either phosphorylated (+) or not (−) in vitro by CDK2 (A) and CDK1 (B), and then subjected to western blot analysis with anti-DLG1 monoclonal antibody (WB DLG1), the anti-Ser442-P and anti-Ser158-P antibodies. The lower panel shows the ponceau stain of the nitrocellulose membrane used for the CDK1 analysis and demonstrates equal levels of protein loading in all lanes. Note equal levels of reactivity with the anti-DLG1 antibody and specific recognition of the phosphorylated forms of DLG1 by the anti-phospho antibodies. (C) HaCaT cells were synchronised with aphidicolin and then released and cell extracts harvested at different phases of the cell cycle. DLG1 was then immunoprecipitated with anti-DLG1 monoclonal antibody, and the pattern of DLG1 expression analysed by western blotting with anti-DLG1 monoclonal antibody (WB DLG1) and the anti-Ser158-P and anti-Ser442-P antibodies. Cell extracts were also analysed using anti-tubulin antibody to confirm equal amounts of protein extraction. Note low level of reactivity of the anti-phospho antibodies in asynchronous (Asyn) and G1-arrested cells and the specific increases in S (5 hours for Ser158, 6.5 hours for Ser442) and M phases (9 hours for Ser158) of the cell cycle. (D) Asynchronously growing HaCaT cells were untreated (−) or treated (+) with roscovitine (50 μM) for 3 hours. DLG1 was then immunoprecipitated with anti-DLG1 monoclonal antibody, and the pattern of DLG1 expression analysed by western blotting with anti-Dlg monoclonal antibody (WB D lg) and the anti-Ser158-P antibody. Cell extracts were also analysed using anti-tubulin antibody to confirm equal amounts of protein extraction. Note the loss in reactivity of the anti-phospho antibody treatment.

Materials and Methods

Cell culture and transfections
HaCaT, U2OS and HEK293 cells were all maintained in DMEM supplemented with 10% fetal calf serum. Cells were transfected using the standard calcium phosphate precipitation method. The inhibitor roscovitine (Calbiochem) was used at 50 μM to synchronise HaCaT cells, aphidicolin (Sigma) was added at a concentration of 4 μg/ml to asynchronous growing cells for 24 hours. The aphidicolin-containing medium was then removed and the cell culture was washed with 10 ml PBS. The PBS was then replaced with complete medium. To achieve M-phase synchronisation, cells were incubated for an additional 40 minutes at 4°C. Protein-A-Sepharose beads (GE Healthcare) were then added to the lysate and incubated with anti-CDK2 polyclonal antibody (Santa Cruz) or anti-cyclin B monoclonal antibody (Calbiochem) for 3-4 hours on a rotating wheel at 4°C. Protein-A-Sepharose beads (GE Healthcare) were then added to the lysate and incubated for an additional 40 minutes at 4°C. The lysates were centrifuged and washed in PBS and trypsinised, in E1A buffer [25 mM HEPES pH 7.0, 0.1% NP-40, 150 mM NaCl, protease inhibitor cocktail I (Calbiochem)]. After incubation on ice for 20 minutes, lysates were cleared by centrifugation at 21,000 g for 10 minutes. SDS loading buffer was then added and the extracts were analysed by SDS-PAGE and western blot assays. For communoprecipitations, endogenous protein was immunoprecipitated from HEK293 cells extracted with E1A buffer. The soluble fraction was incubated with anti-CDK2 polyclonal antibody (Santa Cruz) or anti-cyclin B monoclonal antibody (Calbiochem) for 3-4 hours on a rotating wheel at 4°C. Protein-A-Sepharose beads (GE Healthcare) were then added to the lysate and incubated for an additional 40 minutes at 4°C. The lysates were centrifuged and washed three times with E1A buffer and precipitated proteins were analysed by western blotting. In all cases, endogenous DLG1 was detected using anti-DLG1 monoclonal antibody (2D11, Santa Cruz, Heidelberg, Germany) and HA-tagged DLG was detected using anti-HA monoclonal antibody (2D11, Santa Cruz, Heidelberg, Germany). Immunoprecipitations were analysed using anti-CDK2 and anti-cyclin B antibodies (Santa Cruz). Tubulin was detected using anti-tubulin monoclonal antibody (Sigma) and anti-β-galactosidase antibody (Promega) was used to monitor transfection efficiency. Western blots were developed using the GE Healthcare ECL System according to the manufacturer’s instructions.

GST fusion proteins and in vitro kinase assays
GST fusion proteins were produced and purified as described previously (Thomas et al., 1997) and their purity was tested by SDS-PAGE and Coomassie blue staining. Equal amounts of purified GST proteins were incubated with commercially purified CDK1 and CDK2 kinases (New England Biolabs) for 20 minutes at 30°C in

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phosphorylation buffer (20 mM HEPES, pH 7.5, 20 mM MgCl₂, 0.3 mM aprotinin, 1 mM pepstatin, containing 56 mM [³²P]ATP (Amersham) and 10 mM ATP. After being extensively washed, the phosphorylated proteins were detected by SDS-PAGE and autoradiography.

Immunofluorescence and microscopy
Cells were fixed with 3.7% paraformaldehyde in PBS and permeabilised with 0.1% Triton X-100 in PBS. Primary antibodies were incubated for 1.5 hours at 37°C, followed by extensive washing in PBS and incubation for 30 minutes at 37°C with secondary anti-rabbit or anti-mouse antibody conjugated with fluorescein or rhodamine (Molecular Probes). For visualisation of DNA, cells were stained with Hoechst 33258 stain (Sigma). Samples were then washed several times with water and mounted with Vectashield mounting medium (Vector Laboratories) on glass slides. Slides were analysed with either a Leica DMLB fluorescence microscope equipped with a Leica photo camera (A01M871016) or a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a ×100 or a ×63 objective oil-immersion lens.

Ubiquitylation assays
HEK293 cells were transfected with 2 μg HA-DLG1 or mutant plasmids, and 0.5 μg PCMV-Flag-UBiquitin. 24 hours post transfection, the cells were treated with proteasome inhibitors CBZ (MGI32, 50 μM, Sigma) and LiCl (50 μM, Sigma) for 3 hours, after which HA extraction was performed and the soluble fraction was incubated with anti-HA agarose beads (Sigma) to pull down DLG1 for 1-2 hours on a rotating wheel at 4°C. The agarose beads were then extensively washed and the precipitated proteins were analysed by western blot using the Flag antibody (M2, Sigma) to detect ubiquitin.

Half-life experiments
HEK293 cells were transfected using calcium phosphate precipitation with 2 μg wild type HA-DLG1 or the mutants, along with 0.1 μg of the β-gal plasmid as a transfection control. 24 hours after transfection, the cells were treated for different time points as indicated with cycloheximide (50 μg/ml in DMSO) to block protein synthesis. Total cellular extracts were then analysed by western blot and the intensity of the bands was collected with a ×100 or a ×63 objective oil-immersion lens.

References
Gardiol, D., Zaccii, A., Petrema, F., Stanta, G. and Banks, L. (2006). Human discs large and scrub are localized at the same regions in color mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. Int. J. Cancer 119, 1285-1290.


