Cdk5/p35 phosphorylates lemur tyrosine kinase-2 to regulate protein phosphatase-1C phosphorylation and activity

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Abstract
Cyclin-dependent kinase-5 (cdk5)/p35 and protein phosphatase-1 (PP1) are two major enzymes that control a variety of physiological processes within the nervous system including neuronal differentiation, synaptic plasticity and axonal transport. Defective cdk5/p35 and PP1 function are also implicated in several major human neurodegenerative diseases. Cdk5/p35 and the catalytic subunit of PP1 (PP1C) both bind to the brain-enriched, serine–threonine kinase lemur tyrosine kinase-2 (LMTK2). Moreover, LMTK2 phosphorylates PP1C on threonine-320 (PP1Cthr320) to inhibit its activity. Here, we demonstrate that LMTK2 is phosphorylated on serine-1418 (LMTK2ser1418) by cdk5/p35 and present evidence that this regulates its ability to phosphorylate PP1Cthr320. We thus describe a new signalling pathway within the nervous system that links cdk5/p35 with PP1C and which has implications for a number of neuronal functions and neuronal dysfunction.

Keywords: Alzheimer’s disease, amyotrophic lateral sclerosis, apoptosis-associated tyrosine kinase, axonal transport, kinesin-1, lemur tyrosine kinase-1.


LMTK2 also known as cyclin-dependent kinase-5 (cdk5)/p35 regulated kinase, kinase/phosphatase/inhibitor-2, brain-enriched kinase and apoptosis-associated tyrosine kinase-2, is a member of the lemur family of membrane-associated kinases (Wang and Brautigan 2002; Kesavapany et al. 2003; Kawa et al. 2004). Although originally predicted to be a dual-specificity serine–threonine/tyrosine kinase, several studies have shown that LMTK2 targets only serine and threonine residues (Wang and Brautigan 2002; Kawa et al. 2004).

The functions of LMTK2 are not properly understood. LMTK2 knockout mice are viable but males are infertile because of defects in spermatogenesis (Kawa et al. 2006). However, LMTK2 is ubiquitously expressed and this suggests that it has other physiological roles; in particular, LMTK2 expression is high in the brain where it is found in most neuronal subtypes (Wang and Brautigan 2002; Kesavapany et al. 2003; Kawa et al. 2004). LMTK2 has been implicated in intracellular transport and trafficking, endocytic recycling and nerve growth factor signalling, although the precise mechanisms by which it impacts on all of these different processes remain unclear (Kawa et al. 2004; Chibalina et al. 2007; Inoue et al. 2008; Manser et al. 2011).

One route whereby LMTK2 might influence a variety of cellular functions comes from the finding that it binds to and phosphorylates PP1C (Wang and Brautigan 2002; Manser et al. 2011). PP1C is a major phosphatase that regulates many cellular events (Cohen 2002). LMTK2 binds to PP1C via a valine–threonine–phenylalanine (val–thr–phe) motif (residues 1325–1327 in mouse LMTK2) and induces inhibitory phosphorylation on PP1Cthr320 (Wang and Brautigan 2002; Manser et al. 2011).

The mechanisms that control LMTK2 phosphorylation of PP1C are unknown. However, LMTK2 also binds to cdk5/p35, a kinase that has been implicated in the regulation

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Abbreviations used: cdk5, cyclin-dependent kinase-5; LMTK2, lemur tyrosine kinase-2; PP1C, protein phosphatase-1C; val–thr–phe, valine–threonine–phenylalanine.
of PP1CThr\textsuperscript{320} phosphorylation (Kesavapany \textit{et al.} 2003; Morfini \textit{et al.} 2004). Cdk5/p35 is a major neuronal kinase that controls a diverse array of functions; also, dysregulation of cdk5/p35 has been implicated in several neurodegenerative diseases (Su and Tsai 2011). Cdk5/p35 phosphorylates LMTK2 but the residues targeted and how such phosphorylation influences LMTK2 phosphorylation of PP1CThr\textsuperscript{320} are not known (Kesavapany \textit{et al.} 2003). Here, we demonstrate that cdk5/p35 phosphorylates LMTK2 on serine\textsuperscript{1418} to regulate its ability to phosphorylate PP1CThr\textsuperscript{320}.

\section*{Materials and methods}

\subsection*{Mass spectrometry}
Identification of phosphorylation sites was performed following the immunoprecipitation of LMTK2 essentially as described previously (Vagnoni \textit{et al.} 2011).

\subsection*{Plasmids}
Expression vectors for PP1C, p35, myc-tagged mouse LMTK2 and LMTK2 in which the val–thr–phe PP1C-binding motif (residues 1325–1327) were mutated to ala (LMTK2ala) to inhibit PP1C binding and control pCneo vector containing the Escherichia coli chloramphenicol acetyltransferase were as described (Guidato \textit{et al.} 1996; Manser \textit{et al.} 2011). LMTK2 serine\textsuperscript{1418} was mutated to alanine or aspartate using a QuiikChange XL Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) and the following primer pairs: 5’-CCTCCAAATCTCCTCCCCACCGGGACG-3’ and 5’-CCTGGCCGGTGGGGGAGGAGCG-3’ (LMTK2ser\textsuperscript{1418ala}), and 5’-CCTCCAAAATCTCCTCCCCACCGGGACG-3’ and 5’-GGCCGGTGGAAGGAGGATCGAAGTATTTGGAGG-3’ (LMTK2ser\textsuperscript{1418asp}).

\subsection*{Antibodies}
The following antibodies were used: anti-Myc-tag (9B11) and anti-PP1C (a (Thr320) (Cell Signaling Technology, Danvers, MA, USA); cdk5, p35 and anti-PP1C phospho-PP1C\textsuperscript{a} (Cell Signaling Technology, Danvers, MA, USA); cdk5, p35 and anti-PP1C (E-9) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-tubulin DM1A (Sigma, Gillingham, Dorset, UK). Rabbit LMTK2 antibody was as described (Chibalina \textit{et al.} 2007). Rabbit antibody to phosphorylated LMTK2ser\textsuperscript{1418} was generated by immunization with the synthetic phosphopeptide SLQTSKYFS(phos)PPPPAR coupled to keyhole limpet haemocyanin. Serum was affinity-purified using a phosphopeptide column followed by cross-absorption with non-phosphopeptide (Proteintech, Chicago, IL, USA).

\subsection*{Cell culture and transfection}
Chinese hamster ovary and HeLa cells were grown in Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium, respectively, containing 10% foetal bovine serum supplemented with 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Paisley, UK). Cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturers’ instructions. As a control vector and so that all transfections received the same amounts of DNA, pCneo vector containing the \textit{E. coli} chloramphenicol acetyltransferase was used as a balance. Cortical neurons were obtained from embryonic day 18 rat embryos (Charles River, Margate, Kent, UK) and cultured as described previously (Ackerley \textit{et al.} 2000) and according to Home Office and Institutional guidelines. To inhibit cdk5/p35, neurons were treated with either 25 \textmu M roscovitine or 50 \textmu M olomoucine (Sigma) for 4 h prior to analyses.

\subsection*{Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblotting and immunoprecipitation}
Cells were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting as described (Manser \textit{et al.} 2011). Phosphate was removed from proteins on immunoblots using alkaline phosphatase from \textit{E. coli} (Sigma). Immunoprecipitation assays were performed as described (Vagnoni \textit{et al.} 2011) using antibody 9B11 to immunoprecipitate transfected LMTK2. Signals on immunoblots were quantified as described (Vagnoni \textit{et al.} 2011); PP1CThr\textsuperscript{320} phosphorylation signals were normalized to total PP1C and analysed using one-way ANOVA tests with Bonferroni post hoc test for multiple comparisons.

\section*{Results}
\subsection*{LMTK2 is phosphorylated on serine\textsuperscript{1418}}
To identify putative cdk5/p35 phosphorylation sites in LMTK2, we used mass spectrometry to sequence LMTK2 from CHO cells in which endogenous cdk5 was activated by transfection of p35. Non-neuronal cells, such as the CHO and HeLa cells used in this study, express cdk5 but this is not active because of the absence of the cdk5 activator p35 whose expression is restricted mainly to neurons; transfection of p35 induces cdk5 activity in these cells by activating endogenous cdk5 (Tsai \textit{et al.} 1994; Guidato \textit{et al.} 1996; Li \textit{et al.} 2000). Following trypsin digestion, we obtained 66% sequence coverage of LMTK2 and this was identical to that deposited in the database (NCBI accession number NM_001081109). LMTK2ser\textsuperscript{1418} was identified as a phosphorylated residue within the peptide YFSPPPPAR (Fig. 1). LMTK2ser\textsuperscript{1418} and surrounding sequences are highly conserved between rodent and human LMTK2.

\subsection*{LMTK2ser\textsuperscript{1418} is phosphorylated by cdk5/p35}
LMTK2ser\textsuperscript{1418} precedes a proline and, as such, is targeted for phosphorylation by proline-directed kinases such as cdk5/p35. Indeed, analyses using NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK), which predicts kinases that phosphorylate particular sites in proteins, gave the highest score for the phosphorylation of LMTK2ser\textsuperscript{1418} by cdk5/p35 (score 0.64) (for comparison, glycogen synthase kinase-3\textbeta{} and p38 mitogen activated kinase each gave the next highest scores of 0.52). However, to more formally confirm that LMTK2ser\textsuperscript{1418} is phosphorylated by cdk5/p35, we first generated an antibody that specifically detects LMTK2 phosphorylated on ser\textsuperscript{1418}. By immunoblotting, this antibody detected transfected LMTK2 in HeLa cells but not following the treatment of the blot with alkaline phosphatase to remove phosphate (Fig. 2a). Moreover, this LMTK2ser\textsuperscript{1418} phospho-
antibody had increased reactivity with LMTK2 in cells co-transfected with LMTK2 and p35 compared to cells transfected with LMTK2 alone, and mutation of LMTK2ser1418 to alanine (LMTK2ser1418ala) to block phosphorylation, abolished labelling by this antibody in both LMTK2ser1418ala and LMTK2ser1418ala + p35 co-transfected cells (Fig. 2b). These studies demonstrate the specificity of the LMTK2ser1418 phospho-antibody and show that cdk5/p35 phosphorylates LMTK2ser1418 in transfected cells. To obtain evidence that endogenous cdk5/p35 phosphorylates LMTK2ser1418, we monitored LMTK2ser1418 phosphorylation in cultured rat cortical neurons treated with roscovitine and olomoucine, two structurally different cdk5/p35 inhibitors. Both inhibitors reduced endogenous LMTK2ser1418 phosphorylation without affecting the overall LMTK2 protein levels (Fig. 2c). Together with the mass spectrometry and bioinformatic evidence, these studies demonstrate that cdk5/p35 phosphorylates LMTK2ser1418.

Mutation of LMTK2ser1418 does not affect LMTK2 binding to PP1C but influences LMTK2 phosphorylation of PP1Cthr320

LMTK2ser1418 is situated towards the extreme carboxy-terminus of LMTK2 and, as such, is relatively close to its val–thr–phe PP1C-binding motif [LMTK2ser1418 and the PP1C binding motif are separated by 90 (human) and 91 (mouse) amino acids]. To gain insight into whether LMTK2ser1418 phosphorylation influences the interaction between LMTK2 and PP1C, we used immunoprecipitation assays to monitor the binding of PP1C to LMTK2ser1418ala, a mutant that cannot be phosphorylated, and LMTK2ser1418asp.
a mutant in which ser1418 is mutated to aspartate to mimic permanent phosphorylation. There are many examples where replacing serines with a negatively charged residue such as aspartate accurately mimics the effect of phosphorylation of the site (e.g. Ackerley et al. 2003; Vagnoni et al. 2011). Mutation of LMTK2ser1418 to either alanine or aspartate did not influence LMTK2 binding to PP1C in these assays (Fig. 3a). However, as previously described (Wang and Brautigan 2002; Manser et al. 2011), the LMTK2-PP1C interaction was markedly reduced following mutation of the PP1C-binding motif in LMTK2 (val–thr–phe to ala–ala–ala; LMTK2 ser1325–1327) (Fig. 3a).

We next considered whether the mutation of LMTK2ser1418 influenced the phosphorylation of PP1Cthr320. To do so, we co-transfected HeLa cells with PP1C and either control vector, wild-type LMTK2, LMTK2ser1418ala, LMTK2ser1418asp or the PP1C-binding defective mutant LMTK2 val1325–1327ala, and monitored the phosphorylation of PP1Cthr320 using immunoblots. As previously described (Wang and Brautigan 2002; Manser et al. 2011), transfection of LMTK2 increased PP1Cthr320 phosphorylation and this effect was markedly inhibited in cells co-transfected with the PP1C-binding mutant LMTK2 val1325–1327ala (Fig. 3b). However, co-transfection of LMTK2 ser1418ala decreased whereas co-transfection of LMTK2ser1418asp increased PP1Cthr320 phosphorylation (Fig. 3b). We also investigated the effect of activating cdk5 by transfection of p35, on LMTK2-induced phosphorylation of PP1Cthr320. Here, cells were transfected with PP1C and either LMTK2 + control vector or LMTK2 + p35, and PP1Cthr320 phosphorylation again monitored by immunoblotting. Co-transfection of LMTK2 with p35 increased PP1Cthr320 phosphorylation compared with LMTK2 + control-transfected cells (Fig. 3c). These increases in PP1Cthr320 phosphorylation induced by co-transfection of LMTK2ser1418asp or p35 were less marked than the increase in LMTK2ser1418 phosphorylation induced by p35 transfection (compare Fig. 2b with Fig. 3b and c); this may reflect the properties of the two antibodies used to detect PP1Cthr320 and LMTK2ser1418 phosphorylation. Nevertheless, these findings together show that cdk5/p35 phosphorylation of LMTK2ser1418 stimulates LMTK2-mediated phosphorylation of PP1Cthr320.

**Discussion**

Here, we identify serine1418 as a phosphorylation site in LMTK2. Cdk5/p35 binds to LMTK2 (Kesavapany et al.

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**Fig. 3** Mutation of LMTK2ser1418 to preclude or mimic phosphorylation does not affect LMTK2 binding to PP1C but alters LMTK2 phosphorylation of PP1Cthr320. (a) Immunoprecipitation assays of LMTK2 and PP1C in transfected HeLa cells. Cells were transfected with control vector (Ctrl) or PP1C, or PP1C and either LMTK2, LMTK2 val1325–1327ala (LMTK2ala), LMTK2ser1418ala (LMTK2S1418A) or LMTK2ser1418asp (LMTK2S1418D). LMTK2 was immunoprecipitated (IP) via the myc-tag and the immunoprecipitates probed on immunoblots for LMTK2 and PP1C. Also shown are immunoblots of the input lysates. (b) Mutation of LMTK2ser1418 influences LMTK2 phosphorylation of PP1Cthr320. Cells were transfected with control vector (Ctrl), PP1C and Ctrl, or PP1C and either LMTK2, LMTK2ser1418ala (LMTK2S1418A), LMTK2ser1418asp (LMTK2S1418D) or LMTK2ala as indicated. The samples were then probed on immunoblots for phosphorylated PP1Cthr320 (PP1Cthr320p), total PP1C and LMTK2 via the myc tag. Bar chart shows relative signal intensities for PP1Cthr320 phosphorylation normalized to total PP1C. Error bars are SEM, *p < 0.05, **p < 0.01, ***p < 0.001. (c) p35 stimulates LMTK2 phosphorylation of PP1Cthr320. Cells were transfected with PP1C and either LMTK2 and control vector (LMTK2), or LMTK2 and p35 and probed on immunoblots for phosphorylated PP1Cthr320, PP1C, LMTK2 and p35 as indicated.
and show that the modulation of cdk5/p35 activity alters LMTK2ser1418 phosphorylation. LMTK2 induces inhibitory phosphorylation of PP1Cthr320 (Wang and Brautigan 2002; Manser et al. 2011) and we also show that mutation of LMTK2ser1418 to preclude or mimic phosphorylation regulates LMTK2 phosphorylation of PP1Cthr320. Together, these results are consistent with the notion that cdk5/p35 phosphorylates LMTK2ser1418 to stimulate LMTK2 phosphorylation of PP1Cthr320. However, we cannot eliminate the possibility that LMTK2 recruits other kinases, which may also phosphorylate PP1Cthr320, and that cdk5/p35 influences this process.

We previously demonstrated that cdk5/p35 binds to and phosphorylates LMTK2 but, as no LMTK2 substrates were identified at that time, we conducted autophosphorylation studies to examine LMTK2 activity and these experiments showed that cdk5/p35 inhibited LMTK2 autophosphorylation (Kesavapany et al. 2003). Our new findings refine these initial observations and demonstrate that cdk5/p35 stimulates LMTK2 activity at least in its ability to mediate PP1Cthr320 phosphorylation.

Some of our studies were performed in HeLa cells where cdk5 is present but inactive because of the absence of p35. In such cells, transfection of p35 increased LMTK2ser1418 phosphorylation but we still obtained signals for LMTK2ser1418 phosphorylation using a phospho-specific antibody in cells not transfected with p35 and these signals were abolished after the treatment of the blots with phosphatase (cf. Fig. 2). These results suggest that LMTK2ser1418 is phosphorylated at low levels in HeLa cells in the absence of cdk5/p35 activity. As such, there may be other kinases in HeLa and other non-neuronal cell types that phosphorylate LMTK2ser1418. One possibility is that these as yet unidentified kinases include other members of the cdk family such as those involved in regulating the cell cycle.

Cdks/p35 and PP1C are major enzymes that together control protein phosphorylation to regulate a diverse array of physiological processes within the nervous system. These include nerve growth factor-induced neuronal differentiation (Li et al. 2007), synaptic plasticity (Benavides and Bibb 2004), and kinesin-1 function and axonal transport (Morfini et al. 2004). In addition, both cdk5/p35 and PP1C dysfunction are linked to some neurodegenerative diseases (Morfini et al. 2004; Su and Tsai 2011). The mechanisms by which cdk5/p35 and PP1C impact upon these different processes are not fully clear. For example, cdk5/p35 influences kinesin-1 function via an effect on PP1Cthr320 phosphorylation but cdk5/p35 does not directly phosphorylate PP1Cthr320 in this pathway (Morfini et al. 2004). Our identification of LMTK2ser1418 phosphorylation as a downstream target of cdk5/p35 that regulates LMTK2 phosphorylation of PP1Cthr320 thus provides new insight into the routes by which cdk5/p35 and PP1C might together influence neuronal physiology. Interestingly, LMTK1 (another member of the LMTK family) is also phosphorylated by cdk5/p35 to regulate its activity (Tsutsunimi et al. 2010). Thus, cdk5/p35 may regulate the LMTK family of kinases to influence a number of neurophysiological processes.

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