LIN-9 Phosphorylation on Threonine-96 Is Required for Transcriptional Activation of LIN-9 Target Genes and Promotes Cell Cycle Progression

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Abstract

Cell cycle transitions are governed by the timely expression of cyclins, the activating subunits of Cyclin-dependent kinases (Cdks), which are responsible for the inactivation of the pocket proteins. Overexpression of cyclins promotes cell proliferation and cancer. Therefore, it is important to understand the mechanisms by which cyclins regulate the expression of cell cycle promoting genes including subsequent cyclins. LIN-9 and the pocket proteins p107 and p130 are members of the DREAM complex that in G0 represses cell cycle genes. Interestingly, little is known about the regulation and function of LIN-9 after phosphorylation of p107,p130 by Cyclin D/Cdk4 disassembles the DREAM complex in early G1. In this report, we demonstrate that cyclin E1/Cdk3 phosphorylates LIN-9 on Thr-96. Mutating Thr-96 to alanine inhibits activation of cyclins A2 and B1 promoters, whereas a phosphomimetic Asp mutant strongly activates their promoters and triggers accelerated entry into G2/M phase in 293T cells. Taken together, our data suggest a novel role for cyclin E1 beyond G1/S and into S/G2 phase, most likely by inducing the expression of subsequent cyclins A2 and B1 through LIN-9.

Introduction

Cell cycle transitions are tightly regulated by the timely expression and degradation of cyclins, the regulatory subunits of cyclin-dependent kinases (Cdks). E-type cyclins are exclusively available at early stages of DNA synthesis and a large body of evidence suggests that they are essential to drive G1/S transition [1]. E-type cyclins are found overexpressed in a variety of human cancers and are believed to contribute to oncogenesis [2]. However, they are largely dispensable in normal somatic cells and for mouse development [3], thus making them an attractive target for cancer therapy. As E-type cyclins are dispensable for normal somatic cells but essential for tumor cell proliferation, it is important to understand how E-type cyclins promote cell cycle progression.

A key role of cyclin E1 is the binding and activation of Cdk2, thereby promoting G1/S transition and centrosome duplication [2]. In addition to Cdk2, Cyclin E1 can activate Cdk3, which is structurally closely related to Cdk2 [4–8]. Early reports indicate that Cdk3 plays a unique role in the G1/S transition. For instance, dominant-negative Cdk3-DN induces a G1/S arrest that cannot be overcome by the expression of SV40, while a similar arrest produced by Cdk2-DN can be rescued by SV40 expression but not by Cdk3 [7]. More importantly, the G1 arrest induced by Cdk3-DN can be rescued by simultaneous expression of Cdk3, but not Cdk2 [8]. These observations demonstrate that Cdk3 exerts unique functions in G1/S phase that cannot be compensated by Cdk2. Cdk3 and other G1 Cdks are responsible for the phosphorylation and inactivation of the pocket proteins retinoblastoma (pRB), p107 and p130 [9], which release the inhibition that pRB/E2F1-3a and p107,p130/E2F4-5 exert on many genes required for S-phase entry [10–13]. Additionally, E2Fs are also necessary for the control of mitotic genes. For example, the transcriptional activation of cyclins A and B, and Cdk1 require the coordinated action of E2F1-3a and other transcription factors such as B-Myb [14]. B-Myb is part of a complex that has been termed dREAM (Drosophila RB, E2F And Myb) after a similar complex originally described in Drosophila [15–17] and has been linked to the cell cycle regulation in G0 and S phases. A similar complex termed DRM or MuvB has been described in C. elegans [18], while a complex that includes LIN-9 (Mip40), LIN-37 (Mip40), LIN-52; and LIN-54 (Mip120), termed MCC/LINC...
(Mip Core Complex, LIN Complex, Multivulva Complex), is stable throughout the cell cycle and specifically binds to p107, p130/E2F4 in G0 and B-Myb in S-phase [18–21]. The interaction of the MCC/LINC complex with B-Myb in S-phase is critical for the induction of G2/M genes [19–22]. Recently, it has been shown that the induction of G2/M genes also requires the interaction of this complex with FoxM1 [23]. Interestingly, little is known about post-translational modifications, such as phosphorylation, that may regulate the function of the MCC/LINC. It has been reported that phosphorylation of LIN-52 was important for the regulation of quiescence and that LIN-9 was phosphorylated in vivo [24]. However, the biological role of this post-translational modification of LIN-9 was not investigated. In this report, we investigate the role of LIN-9 phosphorylation in the transcriptional activation of cell cycle genes. We found that LIN-9 is phosphorylated in a cell cycle dependent manner in cycling cells. We identify Cdk3/cyclin E1 as a novel LIN-9 targeting kinase and provide strong evidence for this phosphorylation being essential for promoting cell cycle progression.

Results

LIN-9 is phosphorylated by and associates with cyclin E/ Cdk3

In cycling cells, LIN-9 promotes the transcription of cell cycle genes such as cyclins A2 and B1, thereby facilitating Cdk activation, and promoting cell cycle transitions [19,21]. To test whether LIN-9 is phosphorylated by cell cycle protein kinases, we subjected GST-LIN-9 expressed in bacteria to in vitro kinase assays using a panel of activated protein kinases (Cdk4/cycD3, Cdk4/cycD1, Cdk6/cycD1, Cdk3/cycE1, Cdk2/cycE1, Cdk1/cycA2, Cdk1/cycB1, Cdk3/cycT, Cdk7/cycH, Cdk3/p35, Cdk3/p25). From this panel, Cdk3/cyclin E1 and to a lesser extent Cdk2/cyclin E1, showed strong kinase activity towards GST-LIN-9 (Fig. 1A and data not shown). Given this observation, we sought to investigate whether Cdk3 can phosphorylate LIN-9 under more physiological conditions. As Cdk3 associates with cyclins E and A in proliferating cells [25], we co-transfected 293T cells with Flag-Cdk3 and untagged cyclin E1 or cyclin A2, immunoprecipitated Flag-Cdk3 and associated cyclins using an anti-Flag antibody, and subjected the immunoprecipitated material to Western blots using LIN-9 antibody (upper panel), cyclin E1 antibody (middle panel) and Flag-antibody (lower panel).
LIN-9 because when this Cdk is associated with other cyclins, i.e. cyclin A2, phosphorylation of LIN-9 was very weak.

As Cdk3 in complex with cyclin E1 is the major kinase phosphorylating LIN-9, we tested whether all these proteins can be found in a trimeric complex in cells. Thus, we co-transfected 293T cells with Flag-Cdk3 and GFP-LIN-9 and subjected cell extracts to immunoprecipitations with an anti-Flag antibody followed by Western blot. Figure 1C demonstrates that Flag-Cdk3 immunoprecipitates (lower panel) contained substantial amounts of both endogenous cyclin E1 (middle panel) and GFP-LIN-9 (upper panel). Taken together, these observations indicate that Cdk3/cyclin E1 is the major kinase phosphorylating LIN-9 during the cell cycle and that LIN-9, Cdk3 and cyclin E1 can be found in a trimeric complex in human cells.

Thr-96 in LIN-9 is the major phosphorylation site for Cdk3/cyclin E1

To narrow down the Cdk3/cyclin E1 phosphorylation site in LIN-9 we first produced deletion constructs of GST-LIN-9 in bacteria and subjected them to in vitro kinase assays using Flag-Cdk3/cyclin E1 complexes immunoprecipitated from 293T cells as a source for kinase activity. A truncated form of LIN-9 encoding residues 1-61 (amino acids sequence as reported in [26], although expressed at lower levels than other GST-LIN-9, was not phosphorylated at all (Fig. 2A, lane 5). However, a fragment containing amino acids 1-109 showed strong phosphorylation by Cdk3/Cyclin E1 (lane 4), suggesting that LIN-9 is phosphorylated by this kinase complex at a region between amino acids (aa) 61 and 109. Consistently, LIN-9 deletion constructs containing residues 1-211 (lane 5) and 1-400 (lane 6) were phosphorylated, whereas a construct encompassing aa 111–444 was not (lane 7). Additionally, a construct encompassing aa 85–542 (L9Δ84) was still a good substrate (lane 8). This indicates that the major phosphorylation site for Cdk3/cyclin E1 in LIN-9 resides between aa 85–109.

Cdk/cyclins are proline directed kinases targeting Serine/Threonine-Proline (S/T-P) motives. As an additional approach we mutated every potential Cdk site (S/T-P) in LIN-9 to alanine (A). Potential Cdk sites were S76, T96, T297, S309 and S321. We substituted all these potential Cdk sites in LIN-9 to A, expressed them as GST-LIN-9 proteins in bacteria and subjected them to in vitro kinase assays using Flag-Cdk3/cyclin E1 immunoprecipitated from human cells as kinase source. Of all GST-LIN-9 mutants, GST-LIN-9 T96A showed the weakest incorporation of radiolabel (Fig. 2B, lane 4), consistent with the notion that the major phosphorylation site in LIN-9 resides between aa 85–109.

Knockdown of Cyclin E impairs phosphorylation of Thr-96 of LIN-9 in HUVECs

To test whether Thr-96 in LIN-9 is phosphorylated by Cdk3/cyclin E in vivo, we produced a phospho-specific antibody, recognizing LIN-9 only when phosphorylated on Thr-96 (p-LIN-9Thr-96). First, we tested the p-LIN-9Thr-96 antibody for specificity. As LIN-9 phosphorylation is most prominent when cells are overexpressing Cdk3 and cyclin E (see figure 1B), we co-transfected 293T cells with plasmids encoding GFP-LIN-9, Flag-Cdk3 and cyclin E, to ensure efficient phosphorylation of Thr-96 in LIN-9.

Resulting cell extracts were subjected to immunoprecipitation using our own mouse monoclonal LIN-9 antibody. IP material was split into two, and incubated for 1 hour at 30°C with, or without, lambda phosphatase. Samples were subjected to Western blot using our p-LIN-9Thr-96 antibody. Our p-LIN-9Thr-96 antibody detected a strong signal from the IP material that was incubated without lambda phosphatase (Fig. 3A, first lane), whereas the signal from the sample that was incubated with lambda phosphatase was greatly reduced (Fig. 3A, second lane). This indicates that the phosphatase efficiently dephosphorylated LIN-9 on Thr-96 and that the p-LIN-9Thr-96 antibody is specific. Equal loading of LIN-9 was confirmed by incubating the same membrane with our rabbit antibody against LIN-9 (Fig. 3A, lower panel). We also confirmed the specificity of the p-Thr-96 antibody by using GST-LIN-9 produced in bacteria, phosphorylated by Flag-IP material from cells transfected with Flag-Cdk3/cyclin E in vitro using Flag-Cdk3-DN/cyclin E, as a negative control (data not shown). These data suggest, that our p-LIN-9Thr-96 antibody specifically recognizes LIN-9 when phosphorylated by cyclin E/Cdk3 on Thr-96. Next we sought to investigate whether cyclin E is essential for phosphorylation of Thr-96 of endogenous LIN-9 in primary cells in vivo. Therefore, we transfected primary Human Umbilical Vein Endothelial Cells (HUVEC) with siRNA targeting cyclin E and monitored phosphorylation of LIN-9 on Thr-96 using our p-LIN-9Thr-96 antibody. In cells transfected with control siRNA, pThr-96 of LIN-9 was readily detectable (Fig. 3B, upper panel, first lane). However, in cells transfected with siRNA against cyclin E, phospho-Thr-96 was undetectable (Fig. 3B, upper panel, second lane) indicating that knockdown of cyclin E greatly impairs phosphorylation of LIN-9 on Thr-96 in vivo in primary cells. Western Blot for LIN-9 (second panel), cyclin E (third panel) and tubulin (fourth panel) confirmed equal loading and efficient knockdown of cyclin E in this experiment. In summary, the data obtained by our p-LIN-9Thr-96 antibody indicate that LIN-9 is phosphorylated on Thr-96 in vivo and that cyclin E is essential for mediating this phosphorylation in primary cells.

We next performed cell cycle synchronization experiments using T98G cells followed by immunoblotting with the p-LIN-9Thr-96 antibody to determine the cell cycle distribution of p-LIN-9Thr-96 and to assess the phosphorylation of LIN-9 in tumor cells. Thr-96 phosphorylation of LIN-9 was detected at low levels in G0, and peaked at 8 and 12 hours, a time when cyclin E expression also increased (Fig. 3C). FACS analysis revealed that the subsequent decrease in LIN-9 phosphorylation observed after 12 hours coincides with cells entering S-phase. Interestingly, after
12 hours, a time when phosphorylation of LIN-9 on Thr-96 decreases, expression of cyclin A is induced. Thus, LIN-9 phosphorylation precedes the rise in cyclin E expression, which in turn is followed by cyclin A expression. These data suggest that phosphorylation of LIN-9 is an early G1 event that is likely driven by Cyclin E/Cdk3. It is not surprising that Cyclin E expression continuous to increase beyond the G1/S boundary since it has been proposed that the activation of Cdk2 by this cyclin occurs after the activation of Cyclin E/Cdk3 closer to the G1/S boundary and can still be detected in early S-phase [7,8].

Mutations of Thr-96 of LIN-9 affect the activation of specific promoters

In cycling cells, LIN-9 is known to induce activation of cyclins A2 and B1 promoters [19,21,23,27]. Additionally, we have demonstrated that LIN-9 also binds to the cdc6 promoter, which plays a role in senescence and cell cycle progression [28]. To test whether phosphorylation of LIN-9 on Thr-96 by Cdk3/cyclin E is important for LIN-9-mediated cell cycle progression by inducing transcription of cell cycle genes, we investigated whether mutants that cannot be phosphorylated (LIN-9 T96A) or mimic phosphorylation (T96D) are capable of inducing activation of cell cycle genes. To this end, we performed reporter gene assays using the promoters of cdc6, cyclin A2 and cyclin B1. Wild-type LIN-9 readily induced activation of cdc6, cyclin B1 and cyclin A2 promoters, but non-phosphorylatable LIN-9T96A failed to do so. Moreover, in the case of the cyclin B1 and A2 luciferase reporters, this construct resulted in promoter activation below control levels, suggesting that the T96A mutation has a dominant negative effect by interfering with endogenous LIN-9 function (Fig. 4A). Importantly, LIN-9T96D, mimicking phosphorylation on Thr-96, was much more potent in inducing cdc6, cyclin B1 and cyclin A2 promoter activation than its wild-type counterpart. Taken together, these results strongly suggest that phosphorylation of LIN-9 on Thr-96 is essential for inducing the transcriptional activation of cell cycle genes such as cyclins A2, B1 and cdc6. Noteworthy, the LIN-9-T96D mutant was expressed at much lower levels and migrated faster than both Flag-tagged and GFP-tagged forms in all experiments (Fig. 4B and data not shown). Nevertheless, the low abundance of LIN-9-T96D and the striking enhancement observed for promoter activation of cdc6, cyclin B1 and cyclin A2, it is likely that phosphorylation of LIN-9 on Thr-96 by Cdk3/cyclin E is a key event, triggering cell cycle progression by activating cdc6, cyclins A2 and B1 and possibly other cell cycle genes.
Thr-96 phosphorylation of LIN-9 is important for promotion of S to G2/M transition of the cell cycle

To investigate whether the observed transcriptional activation of cyclins A2, B1 and cdc6 by LIN9 and LIN9T96D (but not LIN9T96A) results in accelerated cell cycle progression, we monitored the cell cycle distribution of 293T cells by FACS. Cells were transfected with the indicated GFP constructs, synchronized by serum starvation, and subsequently released into the cell cycle by serum addition. When cells were analyzed for their cell cycle distribution after transfection of different GFP-LIN-9 constructs, it became evident that transfection of LIN-9 (regardless of Thr96 status) allowed for cells to start synthesis of DNA as indicated by the G1 and G2/M distribution after transfection of different GFP-LIN-9 constructs. GFP-LIN-9T96D migrates faster and is always expressed at lower levels than GST-LIN-9 wild type and T96A. These data strongly suggest that phosphorylation of T96 in LIN-9 triggers cell cycle progression likely through the transcriptional activation of specific cell cycle promoting genes.

Discussion

Recently, a study employing phospho-mass-spectrometry demonstrated that a small fraction of LIN-9 is phosphorylated on Thr-112, a site corresponding to Thr-96 in our system, depending on the ATG considered as start site [24]. The finding of Thr-96 (or Thr-112 respectively) being phosphorylated in vivo strongly supports our notion of Thr-96 being an important phosphorylation site. However, neither the timing nor the kinase responsible for this phosphorylation or the biological relevance of this post-translational modification has been elucidated. In this report, we show that cyclin E/Cdk3 is the kinase responsible for phosphorylation of Thr-96 in LIN-9. Moreover, LIN-9 can be found in a complex with Cdk3 and cyclin E1. All these data strongly suggest that cyclin E/Cdk3 is responsible for the phosphorylation of Thr-96, a site that is phosphorylated in vivo (Litovchick et al., 2011). Thr-96 is also highly conserved among species indicating that this translational modification has biological relevance in vertebrates. The finding that LIN-9 phosphomimetic mutant proteins greatly activate promoters of known LIN-9 targets such as cdc6, cyclin B1 and cyclin A2, indicates that phosphorylation of Thr-96 in LIN-9 promotes cellular activity through transcriptional activation of specific cell cycle promoting genes.
LIN-9 is a key event for transcriptional activation of cell cycle promoting genes. Concomitantly, expression of LIN-9Thr96A inhibited the activation of LIN-9 target promoters below control levels, suggesting a dominant-negative action of this construct. Thus, LIN-9 phosphorylation on Thr-96 is an essential step for the induction of cdc6 and cyclins A2 and B1 and possibly other cell cycle genes. The strong activation of transcription of cdc6, cyclin B1 and A2 promoters is even more dramatic when taken into account that this form of LIN-9 is expressed at much lower levels than its wild type counterpart and the alanine mutant. We hypothesize that the induction of cdc6, cyclins A2 and B1 promoters translates into an accelerated cell cycle progression. In line with this notion, we found expression of the LIN-9Thr96D mutant triggered massive entry of cells into G2/M phase as early as 6 hours after release from quiescence. These results indicate that cyclin E/Cdk3 targets Thr-96 in LIN-9 for phosphorylation and this event triggers activation of cell cycle promoting genes such as cdc6, cyclin A2 and cyclin B1 and promotes S-phase progression and entry into G2/M phase. Interestingly, overexpression of all forms of LIN-9, wild type and mutants, impair the synchronization of 293T cells in G0/G1 suggesting that phosphorylation of LIN-9 – while being essential for S-phase progression – is dispensable for G0/G1 transition, at least in 293T cells. Importantly, the decrease in p-LIN-9Thr96 phosphorylation observed at the G1/S boundary, when LIN-9 is already bound to G2/M promoters [21], suggest that the mechanism of action of p-LIN-9Thr96 may be more complex than the simple activation of target genes within the context of the DREAM complex. For example, phosphorylation of LIN-9 on Thr96 may stabilize the complex on the promoter or recruit other transcriptional players. This would explain why LIN-9 is associated with the Cyclin A and B promoters before B-Myc and prior to their transcriptional activation [21].

In summary, we provide novel insights into the mechanism by which cyclin E controls the expression of subsequent cyclins through LIN-9. Furthermore, our findings suggest a novel mechanism by which cyclin E/Cdk3 promotes cell cycle transitions beyond G1/S phase and into S/G2 phase by triggering the expression of subsequent cyclins A and B through LIN-9. These findings are important because they elucidate the mechanism by which cyclin E passes on the torch to cyclins A and B, to promote timely cell cycle transitions.

**Materials and Methods**

**Cell culture**

T98G and 293FT cells (obtained from ATCC and Life Technologies, respectively) were grown in DMEM containing 10% FBS and 2 mM L-glutamine at 37°C with 5% CO2. HUVECs and endothelial cell growth medium were purchased from Lonza (Walkersville, MD). For synchronization, cells were serum starved for 36 hours and released into the cell cycle by addition of 10% FBS. Cells were transfected using Turbofect (Thermo Scientific) according to the manufacturer’s instructions.

**Constructs and protein purification**

GFP-LIN-9, GST-LIN-9 deletion constructs, and luciferase-constructs under the control of the promoters of cdc6, cyclin B1 or cyclin A2 have already been described [21,28]. Untagged pCMV-cyclin E1 and pCMV-cyclin A2 constructs were a gift from Dr. P Kaldis. Flag-Cdk3 and Flag-LIN-9 constructs were purchased from GeneCopoeia. GST- and GFP-LIN-9 mutants, and Flag-Cdk3-DN were generated by site-directed mutagenesis as described previously [29]. Expression, purification and dialysis of GST-proteins have been described previously [30].

**Antibodies and immunoprecipitation**

Antibodies for LIN-9 have been described previously [26] and the anti-phosphoThr96 was developed by ProteinTech, Inc (Chicago, IL). Antibodies for cyclin E1, cyclin A2 and GFP were from Santa Cruz, antibody for Flag was from Sigma. For IP, cells were lysed in NP-40 lysis buffer (300 mM NaCl, 50 mM Tris pH 8.0, 1 mM MgCl2, 0.2% NP-40, 10% Glycerol, Protease- and Phosphatase-Inhibitors from Roche and Sigma, respectively). For Co-IPs, cell lysis was performed in NP-40 lysis buffer, followed by IP in TIF-buffer (150 mM NaCl, 20 mM Tris pH 8.0, 1 mM MgCl2, 0.1% NP-40, 10% Glycerol and Protease- and Phosphatase-inhibitors from Roche and Sigma, respectively).
Kinase assays and lambda phosphatase assay

Kinase assays using both recombinant kinases from ProQinase (Freiburg, Germany) or Flag-Cdk3 kinase complexes, immunoprecipitated from 293T cells, were performed as described previously [31] with the exception, that kinase reactions were performed in a kinase buffer containing 60 mM HEPES pH7.5, 3 mM MgCl₂, 3 mM MnCl₂, 1.2 mM DTT, 100 cold μM ATP, 2 μCi of [(γ-32P)]ATP at 30°C for 10 min, if not indicated otherwise. For lambda phosphatase assay, 200 U of lambda phosphatase (NEB) were used per IP in 1× NEBuffer supplemented with 1 mM MnCl₂ for 1 hour at 30°C according to the manufacturer’s instructions.

Reporter gene assays

Reporter gene assays were performed as described previously [32], with the exception that cells were plated and transfected on 6 well dishes and cell lysis and analysis was performed using the Promega Glo (Promega) system, following the manufacturer’s instructions.

Supporting information

Figure S1 FACS histograms of the data described in Figure 5.

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Author Contributions

Conceived and designed the experiments: ORC. Performed the experiments: FE MP-N. Analyzed the data: FE ORC. Contributed reagents/materials/analysis tools: ORC. Wrote the paper: FE ORC.

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