A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2–Dpb11

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Phosphorylation often regulates protein–protein interactions to control biological reactions. The Sld2 and Dpb11 proteins of budding yeast form a phosphorylation-dependent complex that is essential for chromosomal DNA replication. The Sld2 protein has a cluster of 11 cyclin-dependent kinase (CDK) phosphorylation motifs (Ser/Thr–Pro), six of which match the canonical sequences Ser/Thr–Pro–X–Lys/Arg, Lys/Arg–Ser/Thr–Pro and Ser/Thr–Pro–Lys/Arg. Simultaneous alanine substitution for serine or threonine in all the canonical CDK-phosphorylation motifs severely reduces complex formation between Sld2 and Dpb11, and inhibits DNA replication. Here we show that phosphorylation of these canonical motifs does not play a direct role in complex formation, but rather regulates phosphorylation of another residue, Thr84. This constitutes a non-canonical CDK-phosphorylation motif within a 28-amino-acid sequence that is responsible, after phosphorylation, for binding of Sld2–Dpb11. We further suggest that CDK-catalysed phosphorylation of sites other than Thr84 renders Thr84 accessible to CDK. Finally, we argue that this novel mechanism sets a threshold of CDK activity for formation of the essential Sld2 to Dpb11 complex and therefore prevents premature DNA replication.

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Introduction

Protein–protein interactions are essential for biological reactions, and in eukaryotic cells are often regulated by protein

modifications, such as phosphorylation. Two yeast replication proteins, Dpb11 and Sld2, form a phosphorylation-dependent complex, then associate with replication origins and further promote loading of DNA polymerases onto the origins to initiate chromosomal DNA replication when cyclin-dependent kinase (CDK) activity increases at the G1/S cell cycle boundary (Kamimura *et al*, 1998; Masumoto *et al*, 2000, 2002).

The Dpb11 protein (Araki *et al*, 1995), and its possible counterparts, Cut5/Rad4 (Fenech *et al*, 1991; Saka and Yanagida, 1993) in fission yeast and TopBP1/Mus101/Cut5 (Yamane *et al*, 1997; Yamamoto *et al*, 2000; Mäkinen *et al*, 2001; Van Hatten *et al*, 2002; Hashimoto and Takisawa, 2003) in higher eukaryotes, constitute a BRCT-domain-rich protein family that functions for DNA replication, repair and cell cycle checkpoints (Garcia *et al*, 2005). In these proteins, BRCT domain occurs as a tandem repeat: recently identified as a phosphopeptide-binding domain (Manke *et al*, 2003; Rodriguez *et al*, 2003; Yu *et al*, 2003; Glover *et al*, 2004). Structural analyses revealed that the phosphopeptide binds to a cleft formed by the interface of these tandem BRCT repeats (for a review, see Glover *et al*, 2004).

The Sld2 protein has 11 clustered CDK-phosphorylation motifs (Ser/Thr–Pro), six of which match the canonical sequences Ser/Thr–Pro–X–Lys/Arg, Lys/Arg–Ser/Thr–Pro and Ser/Thr–Pro–Lys/Arg (Pearson and Kemp, 1991) (Figure 1A and B), so that it is hyperphosphorylated in a CDK-dependent manner (Masumoto *et al*, 2002). Proteomic studies have shown that Sld2 is an excellent substrate of S-phase CDK (Ubersax *et al*, 2003; Archambault *et al*, 2004; Loog and Morgan, 2005). These observations are consistent with Sld2 function for the onset of the S phase. Furthermore, simultaneous substitution of alanine for serine or threonine in all the canonical motifs severely reduces complex formation between Sld2 and Dpb11 and inhibits the initiation of DNA replication: thus, we suggested that CDK-dependent phosphorylations at these sites are essential for the interaction with Dpb11 (Masumoto *et al*, 2002). Sld2 is well conserved in yeasts and fungi (Figure 1A and B), whereas a good candidate for Sld2 in higher eukaryotes had not been reported. Recently, it was proposed that RECQL4 (Supplementary Figure 1A), mutated in Rothmund–Thomson syndrome, is a homologue of Sld2 in vertebrates, because it has weak homology to Sld2 and functions for the initiation of DNA replication in *Xenopus* egg extracts (Sangrithi *et al*, 2005).

CDK is a key regulator of the cell cycle; its activity fluctuates, but peaks at the G1/S and G2/M boundaries. When its activity increases beyond the thresholds at these boundaries, CDK triggers the onsets of the S and M phases. In budding yeast, CDK is composed of three subunits: a catalytic subunit, Cdc28; a Cks1 subunit; and one of nine cyclins, Cln1–Cln3 and Clb1–Clb6 (Mendenhall and Hodge, 1998).

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Figure 1 The interaction regions of Sld2 and Dpb11. (A) Sld2 family proteins in yeast and fungi. Vertical black and grey lines show canonical and non-canonical CDK-phosphorylation motifs, respectively. The shaded boxes indicate the regions containing a cluster of CDK-phosphorylation motifs. Asterisks on boxes indicate Thr84 and Ser100 in Sld2 (Sc) and corresponding residues in Sld2 homologues. Sc, *Saccharomyces cerevisiae*; Eg, *Eremothecium gossypii*; Ca, *Candida albicans*; Sp, *Schizosaccharomyces pombe*; Nc, *Neurospora crassa*. (B) Schematic representation of CDK-phosphorylation motifs in Sld2. The amino-acid sequence required for binding to Dpb11 is shown with the corresponding sequence of Sld2 homologues in different species. Identical amino acids (grey shading) and CDK-phosphorylation motifs (asterisks) are highlighted. (C) The region of Sld2 interacting with Dpb11 in a two-hybrid assay. Various fragments derived from *SLD2* were cloned into pBTM116 (Bartel and Fields, 1995). The resultant plasmids and pACT2-DPB11 (Kamimura *et al*, 1998) were introduced into the yeast strain L40 and the interaction was detected by *lacZ* expression in colour. (D) The interaction region of Dpb11 with Sld2 in a two-hybrid assay. Various fragments of *DPB11* were cloned into pACT2 (Bai and Elledge, 1997) and used for a two-hybrid assay with pBTM116-SLD2 (Kamimura *et al*, 1998) as described in (C).

In general, CDK works with Cln cyclins in the G1 phase, with Clb5 and Clb6 in the G1/S boundary and S phase and with Clb1-Clb4 in the G2/M boundary and M phase.

CDK has a dual role at the onset of S phase. The six-subunit origin recognition complex (Orc) binds throughout the cell cycle at replication origins in budding yeast. From late M phase to G1 phase when the CDK activity is low, a putative replicative helicase, the mini-chromosome maintenance (Mcm) complex (Mcm2-7) is loaded onto replication origins and then forms the pre-replicative complex (pre-RC). This step requires Orc, Cdc6 and Cdt1. The Sld3-Cdc45 complex also associates with origins and its association depends on the pre-RC (Aparicio *et al*, 1999; Zou and Stillman, 2000; Kamimura *et al*, 2001). When CDK and Cdc7 kinase are activated, the three DNA polymerases, Pals α , δ and ϵ , essential for chromosomal DNA replication, are recruited to origins to initiate DNA synthesis with the aid of many replication proteins including Sld2, Dpb11 and GINS (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Kubota *et al*, 2003; Mendez and Stillman, 2003; Takayama *et al*, 2003). Although the initiation of chromosomal DNA replication needs CDK activity absolutely, positive CDK targets to initiate DNA replication have not been identified, except for Sld2. On the other hand, CDK also functions for preventing formation of the pre-RC to ensure that chromosomal DNA replicates once per cell cycle, by phosphorylating each component of the pre-RC: namely Cdc6, Orc, Cdt1 and Mcms (Diffley, 2004; Blow and Dutta, 2005). Cdc6 is degraded by the ubiquitin-proteasome system: free Mcm and Cdt1 are exported from the nucleus: Orc seems to lose activity for pre-RC formation. In addition, a mitotic cyclin, Clb2, binds and inactivates phosphorylated Cdc6 in G2 and the early M phase (Mimura *et al*, 2004).

Although CDK-dependent phosphorylation of Sld2 is essential for complex formation with Dpb11, a precise role of such phosphorylations in complex formation between Sld2 and Dpb11 has not been elucidated. We therefore analysed this complex formation *in vivo* as well as *in vitro* using purified proteins and found, surprisingly, that most of the CDK-phosphorylation motifs do not play direct roles in interaction with Dpb11, but rather regulate phosphorylation of a specific site. We discuss the implications of this hitherto unsuspected mechanism for regulation of the initiation step in chromosomal DNA replication and further argue that this mechanism may be prevalent in phosphorylation-dependent reactions.

Results

A phosphorylated 28-amino-acid stretch of Sld2 binds to Dpb11

To examine complex formation between Sld2 and Dpb11, we first set up an *in vitro* protein-protein binding assay. In a two-hybrid assay, Sld2-P1 (residues 79-263), which contains all the CDK-phosphorylation motifs, interacted with Dpb11 (Figure 1C; P1) and a C-terminal portion of Dpb11 (Dpb11-C; residues 291-631) containing a pair of BRCT domains interacted with Sld2 (Figure 1D). We thus expressed the Sld2-P1 protein and the Dpb11-C protein composed of the C-terminal portion of Dpb11 fused to glutathione S transferase (GST) in *Escherichia coli* and purified them (Supplementary Figure 2). When we phosphorylated Sld2-P1 with recombinant Cdc28-Clb5 (Mendenhall and Hodge, 1998) (rCdc28-Clb5), the yeast S-phase-specific CDK prepared from *E. coli*, Sld2-P1 migrated slower than the unphosphorylated form in

SDS–polyacrylamide gel electrophoresis (PAGE) and bound to the Dpb11-C protein. In contrast, unphosphorylated Sld2-P1 bound to Dpb11-C poorly (Figure 2A), as observed for the full-length proteins *in vivo* (Masumoto *et al*, 2002). We therefore concluded that this *in vitro* assay mimics an *in vivo* reaction fairly. This result further suggests that phosphorylated Sld2 and Dpb11 can form a complex in the absence of other proteins.

The two-hybrid assay indicated that Dpb11 interacts with a 28-amino-acid stretch that has two CDK-phosphorylation motifs (Figure 1C; P7). We therefore challenged the *in vitro* assay with the 28-amino-acid peptide. Although the unphosphorylated peptide (28-NP) did not compete with phosphorylated Sld2-P1 for binding to Dpb11-C, the peptide with phosphorylated residues at CDK-phosphorylation motifs (28-2P) competed efficiently (Figure 2B and C). Moreover, the phosphorylated 28-amino-acid peptide (28-2P) bound to Dpb11-C more efficiently than did the unphosphorylated peptide (28-NP) (Figure 2D), and the phosphorylated Sld2-P1Δ28 protein lacking this 28-amino-acid stretch did not bind to Dpb11-C (Figure 2E). Thus, Dpb11 appears to bind to the 28-amino-acid stretch of Sld2 in a phosphorylation-dependent manner.

Phosphorylation of Thr84 is essential for binding to Dpb11, cell growth and DNA replication

As the 28-amino-acid stretch has two CDK-phosphorylation motifs, we first determined which phosphorylation is responsible for binding to Dpb11 by challenging the *in vitro* assay with various peptides. A 28-amino-acid peptide with phosphorylated Thr84 (pThr84) and with unphosphorylated Ser100 (28-p84) competed with phosphorylated Sld2-P1 for binding, whereas phosphorylation of Ser100 alone (28-p100) did not have the same effect (Figure 2B and F). Consistent with this observation, a 20-amino-acid peptide containing pThr84 but lacking Ser100 (20-p84) competed with phosphorylated Sld2-P1 for binding to Dpb11-C (Figure 2B and F). Thus, while pSer100 is dispensable for this binding, pThr84 is essential.

As we had not been aware of this phosphorylation of Thr84, we confirmed it *in vivo* using anti-pThr84 antibodies (Figure 3A). When cells were arrested in G1 phase by α -factor and released from G1 arrest, the slow migrating form of Sld2, which corresponds to a hyperphosphorylated form, was observed in SDS–PAGE, as reported (Masumoto *et al*, 2002). The Thr84-phosphorylation occurred at the same time as the slow migrating form appeared. However, neither the slow

Figure 2 The 28-amino-acid stretch with pThr84 in Sld2 interacts directly with Dpb11. (A) Complex formation between Flag-Sld2-P1 (residues 79–263) and GST-Dpb11-C (residues 291–631) *in vitro*. Flag-Sld2-P1 (18 pmol) was incubated with 36 units of rCdc28–Clb5 in the presence (+) or absence (–) of ATP at 30°C for 1 h and serial dilutions were further incubated with GST-Dpb11-C (30 pmol) or GST (30 pmol) proteins immobilized on GSH-Sepharose beads at 4°C for 1 h. Sld2-P1 bound to beads was detected by Western blotting with monoclonal anti-Flag antibody (M2, Sigma Aldrich) after SDS–PAGE. The asterisk indicates the slower migrating phosphorylated form of Sld2-P1. (B) Peptide sequence used for the assay. Thick and thin underlines of Sld2-P1 sequence indicate canonical and non-canonical CDK-phosphorylation motifs, respectively. The 28-amino-acid stretch required for binding to Dpb11 is shaded. ‘P’ indicates phosphorylated residues. (C,F) Peptide competition for Sld2-P1 binding to Dpb11. Various peptides shown in (B) were mixed with phosphorylated Flag-Sld2-P1 (0.1 μM) and GST-Dpb11-C (30 pmol) immobilized beads and further incubated at 4°C for 1 h. (D) Biotinylated 28-2P or 28-NP peptides (250 pmol) in (B) immobilized on streptavidin beads were incubated with indicated concentrations of GST-Dpb11-C at 4°C for 30 min and the protein bound to the peptides was detected by Western blotting with anti-GST monoclonal antibody. (E) No binding was observed of Flag-Sld2-P1Δ28 (residues 107–263) lacking the 28-amino-acid binding stretch to the GST-Dpb11-C protein. Reactions were carried out as described in (A), except that Flag-Sld2-P1Δ28 was used instead of Flag-Sld2-P1.

Figure 3 Thr84 of Sld2 is phosphorylated in a CDK-dependent manner and this phosphorylation is essential for cell growth. (A) *YK3* (Δ *sld2*) cells bearing YCpSLD2 (Kamimura *et al*, 1998) (–) or YCpSLD2-10FLAG (+) were arrested in S phase by 0.2 M hydroxyurea (HU) and disrupted by glass beads. The Sld2-10Flag protein was precipitated with anti-Flag antibody M2 from cell lysates. The precipitated proteins were separated using SDS-PAGE and subjected to Western blotting with anti-pThr84 and anti-Flag M2 antibodies. The asterisk indicates the phosphorylated Sld2-10Flag. (B) YS125 (a *GAL-SIC1ΔANT*) and its parental strain W303a harbouring YCp-Gal-SLD2-10FLAG were arrested at G1 phase by α factor and released in YPGal as described (Masumoto *et al*, 2002). The samples were withdrawn at the indicated time and budding cells were counted under a microscope. The Sld2-10Flag protein was precipitated from disrupted cells and analysed as described in (A). (C) *YK3* (Δ *sld2*) (*YEp195SLD2*) (Kamimura *et al*, 1998) cells were transformed with YCplac22 (Vector) or YCpSLD2 bearing the indicated mutation. The resulting transformants were cultivated in YPGA medium and spotted onto plates containing 5-fluoroorotic acid (FOA), as described (Masumoto *et al*, 2002), after serial dilutions. The numbers above the photograph indicate the estimated number of cells placed on a spot. (D) *Y999* (*drc1-1*; *drc1-1* is a temperature-sensitive allele of *SLD2*) (Wang and Elledge, 1999) cells bearing one of YCplac22 (Vector), YCp22SLD2 (WT) and YCp22sld2T84A (T84A) were arrested in G1 and released from G1 at 36°C. Cells were withdrawn at the indicated times and DNA content was measured by flow cytometry. The percentages of budded cells are also shown. (E) The Sld2-10Flag was precipitated from HU-arrested HMS65 (*pep4Δ::G418^rdrc1-1 DPB11-9myc*) cells harbouring YCp22SLD2-10FLAG (WT) or YCp22SLD2-10FLAG bearing a T84A mutation (T84A) as described in (A). Co-precipitation of Dpb11-9myc was detected by anti-myc 9E10 antibody. Note that Sld2-10Flag (T84A) migrated slower than that arrested in G1 phase, as did the WT.

migrating form nor Thr84-phosphorylation was observed in cells expressing a CDK inhibitor, Sic1ΔANT (stable form) (Labib *et al*, 1999) (Figure 3B). Moreover, rCdc28–Clb5 phosphorylated the Thr84 of Sld2-P1 *in vitro* (Figure 5). These results strongly suggest that CDK phosphorylates Thr84 *in vitro* and *in vivo*.

We also tested whether a pThr84 residue is essential for Sld2 function. For this purpose, we substituted an alanine for Thr84. The resultant T84A mutant could not substitute for the wild-type (WT) *SLD2* gene, nor could it promote DNA replication (Figure 3C and D). Furthermore, although the T84A protein showed the slow migrating form in SDS-PAGE, it did not interact with Dpb11 in a two-hybrid assay or co-precipitate with Dpb11 (Figures 3E and 4A). On the contrary, a phosphomimetic aspartate substitution of Thr84 (T84D) allowed both the interaction between Sld2 and Dpb11 in a two-hybrid assay and cell growth (Figures 3C and 4A). Thus, phosphorylation of Thr84 is essential for cell growth and DNA replication, as well as for the *in vivo* interaction between Sld2 and Dpb11.

Sequence specificity of the 28-amino-acid stretch for binding to Dpb11

Most of the *sld2* mutations change various amino-acid residues of the 28-amino-acid stretch, suggesting that amino-acid sequence in this stretch is important (Kamimura *et al*, 1998). We therefore challenged the *in vitro* assay with other variants of the peptide to investigate whether specific amino-acid residues might play an important role in the interaction with Dpb11.

Although a 20-amino-acid peptide containing pThr84 (20-p14) competed efficiently with phosphorylated Sld2-P1, a 14-amino-acid peptide (14-p84) that contains pThr84 but lacks six C-terminal residues reduced competition activity (Figure 2B and F). Furthermore, a 20-amino-acid peptide with pThr84 and an aspartate substitution of a glycine at the 89th residue that corresponds to the *sld2-1* mutation (Kamimura *et al*, 1998) (20-p84D89) (Figure 2B and F) also reduced its activity. Thus, these residues affect the binding to Dpb11.

Position of a phosphorylated residue also affected binding activity to Dpb11. The temperature-sensitive *sld2-6* mutation

Figure 4 Phosphorylations other than Thr84 affect the phosphorylation of Thr84 *in vivo*. (A) The *sld2* mutations at CDK-phosphorylation motifs used for two-hybrid assays and for plasmid-shuffling assays to examine the ability to support cell growth. For a two-hybrid assay, pACT2-Dpb11 (Figure 1D; Dpb11) (Kamimura *et al*, 1998) and pBTM116-SLD2-P1 (Figure 1C; P1) bearing the indicated mutation were used as described in the legend to Figure 1C. Plasmid-shuffling assays were performed as shown in (B) and Figure 3 (C). Brackets in the growth columns indicate results reported previously (Masumoto *et al*, 2002). Note that 6A was previously designated All-A. (B) YST387 (*Δsld2::TRP1* (YEpl95SLD2)) were transformed with YCplac111 (Vector) or YCp111SLD2 bearing the indicated mutation. The resulting transformants were streaked onto an FOA plate. A few colonies carrying SLD2-6A on plasmid showed up in the plasmid-shuffling assay, probably because of the increased copy of mutant proteins. (C) The LexA-fused Sld2-P1 and its variant proteins were precipitated with an anti-LexA antibody (Santa Cruz) from HU-arrested cells carrying pBTM116-SLD2-P1 and its variants shown in (A). The precipitated proteins were analysed by Western blotting using anti-pThr84 and anti-LexA antibodies. The asterisk indicates the slower migrating form of the protein.

replaced Thr-Pro-Gln by Thr-Ser-Gln in the stretch, creating a phosphoinositide-3-kinase-related protein kinase (PIKK) phosphorylation motif Ser-Gln, while the CDK-phosphorylation motif Thr-Pro was lost (Kamimura *et al*, 1998). A novel Ser-Gln motif might be phosphorylated by Mec1 or Tel1, yeast PIKKs, and the interaction between Sld2 and Dpb11 is weakened *in vivo* (Kamimura *et al*, 1998) and *in vitro* (data not shown), whereas a Thr-Gln-Gln substitution for

Thr-Pro-Gln (P85Q) in the stretch allows for interaction with Dpb11 and for cell growth (see below). Furthermore, a 20-amino-acid peptide with a phosphorylated serine substitution at Pro85 (20-p85), corresponding to the *sld2-6* mutation, reduced competition activity, while a peptide with a glutamine substitution at Pro85 and pThr84 (20-p84Q85) competed efficiently (Figure 2B and F). Thus, the relative position of phosphorylated residue seems to be important for the peptide to bind to Dpb11. Taken together, Dpb11 appears to bind to a phosphorylated amino-acid stretch of Sld2 in a sequence-dependent manner, although pThr84 in this stretch is a key determinant for this binding. This is consistent with previous observations in various BRCT domains: some of the amino-acid residues following a phosphorylated serine/threonine play an important role in binding BRCT domains (Manke *et al*, 2003; Rodriguez *et al*, 2003).

The sequence-dependent binding of Dpb11 further specifies the partner. The 20-amino-acid stretch is one of the most conserved regions among Sld2 homologues (Figure 1B). Moreover, the P112S mutation in Drc1, the Sld2 homologue in fission yeast, corresponding to the *sld2-6* mutation described above, confers defective cell growth (Noguchi *et al*, 2002). Thus, we suggest that a binding domain must be conserved in this stretch, even in different organisms. We therefore replaced the amino-acid sequence between Thr84 and Ser100 with the corresponding sequence of Drc1. While Sld2 did not interact with the fission yeast Dpb11 homologue Cut5, the resultant hybrid Sld2 interacted with Cut5 instead of Dpb11 in a two-hybrid assay (Supplementary Figure 3). Thus, a similar region of Sld2 is employed even in different species for binding to Dpb11 homologues, and the sequence between Thr84 and Ser100 specifies the binding partner.

CDK-catalysed phosphorylation at sites other than Thr84 is required for phosphorylation of Thr84

We suggested previously that CDK-phosphorylation motifs in Sld2 are functionally redundant (Masumoto *et al*, 2002). We thus examined whether phosphorylations at positions other than Thr84 affect the interaction with Dpb11 by introducing alanine substitutions. As shown in Figure 4A, a simultaneous alanine substitution of serine or threonine residues at all the canonical CDK-phosphorylation motifs (6A) abolished interaction with Dpb11 (Figure 4A). Moreover, a single alanine substitution at Ser100 (S100A) reduced the interaction. However, phosphorylation of Ser100 is not a single determinant for the interaction, because a five-alanine substitution at the canonical CDK motifs except for Ser100 (5A-1) reduced the interaction. Furthermore, neither single alanine substitution at Ser208 (S208A) nor Thr241 (T241A) reduced the interaction as much as did 5A-1 (Figure 4A). Therefore, phosphorylations at positions other than Thr84 seem to work in concert to promote the binding of Sld2 to Dpb11.

To explain why phosphorylations of not only Thr84 but also other CDK motifs are necessary for the interaction, we hypothesized that phosphorylation of Thr84 requires prior phosphorylations of other residues within Sld2. If this is the case, Sld2 with pThr84 might interact with Dpb11, irrespective of other phosphorylations. We therefore combined several alanine substitutions with an aspartate substitution of Thr84 (T84D) that mimics phosphorylation. Strikingly, substitution of aspartate for Thr84 restored the cell growth defect produced by mutating all the canonical CDK-phosphorylation

motifs, and by mutating all other CDK sites in Sld2 (Figure 4A and B; D84-6A and D84-10A). Thus, it seems likely that CDK phosphorylations other than Thr84 work through phosphorylation of Thr84 and that Sld2 might be accessible to Dpb11, but only once CDK has phosphorylated Thr84. To test this hypothesis more directly, we examined the phosphorylation of Thr84 in several constructs used for the two-hybrid assay. When cells were arrested in S phase by hydroxyurea, LexA-Sld2-P1 was hyperphosphorylated and migrated slowly in SDS-PAGE. This was also true for the T84A and S100A constructs, but not for the 6A and 5A-1 constructs. Thr84 in the LexA-Sld2-P1 construct (Figure 1C) was phosphorylated (Figure 4C; WT), whereas alanine substitutions reduced the phosphorylation of Thr84 (Figure 4C; T100A, 6A, 5A-1). This result strongly suggests that phosphorylations of other sites are required for the phosphorylation of Thr84.

Thr84 phosphorylation is regulated by phosphorylations at sites other than Thr84

To analyse this phosphorylation of Thr84 in detail, we monitored phosphorylation at several CDK-phosphorylation motifs *in vitro* using phospho-specific antibodies. As shown in Figure 5A, rCdc28-Clb5 phosphorylated Thr84 later than it did Ser100 and Thr208. Moreover, while the phosphorylation level of Ser100 increased linearly as the CDK activity increased, the phosphorylation level of Thr84 increased abruptly when the CDK activity increased beyond a specific point (Figure 5B). These results are consistent with the requirement of prior phosphorylation of other sites for the phosphorylation of Thr84, and further suggest that multisite phosphorylation of Sld2 sets a high threshold of CDK activity for the phosphorylation of Thr84.

To exclude the possibility that alanine substitutions at the canonical CDK-phosphorylation motifs confer an unusual conformation on Sld2, we substituted a glutamine for a proline at the 85th residue (P85Q) to convert the CDK-phosphorylation motif, Thr-Pro, to a PIKK phosphorylation motif (Kim *et al*, 1999; O'Neill *et al*, 2000), Thr-Gln, without any change at other CDK-phosphorylation motifs. The P85Q mutant cells grew, as well as did the WT cells, although DNA replication was delayed slightly (Figure 6A and B). Thr84 followed by a glutamine in this construct is probably phosphorylated *in vivo* by either Mec1 or Tel1 kinases (PIKKs in budding yeast) that recognize Ser/Thr-Gln, thus allowing binding to Dpb11 (see Discussion).

We then employed one of the PIKKs, a DNA-dependent kinase (DNA-PK), for *in vitro* phosphorylation of the P85Q construct of Sld2-P1, which contains a single PIKK-phosphorylation motif, Thr⁸⁴-Gln⁸⁵ (Figure 6C). We first incubated T84A and P85Q constructs of Sld2-P1 with rCdc28-Clb5. After incubation, both the constructs migrated slower than the unphosphorylated Sld2-P1, irrespective of DNA-PK, in SDS-PAGE (Figure 6D, lanes 4, 5, 9 and 10), indicating that these constructs were phosphorylated by CDK. Furthermore, when DNA-PK were added to the reaction together with the CDK-inhibitor purvalanol A, the anti-pThr84 antibodies detected phosphorylation of the T85Q construct, but not of the T84A construct (Figure 6D, lanes 5 and 10). Thus, the anti-Thr84 antibodies can detect the phosphorylation of Thr84, followed by glutamine, and DNA-PK phosphorylates Thr84 of the T85Q construct (Figures 6D, lanes 4 and 5). Moreover, this phosphorylation depended on prephosphorylation by

Figure 5 Phosphorylation of Thr84 is regulated by other phosphorylations *in vitro*. **(A)** Time course of phosphorylations at three CDK motifs. Sld2-P1 (45 pmol) was mixed with rCdc28-Clb5 (55 units) in 80 μ l buffer and aliquots were withdrawn at 20-min intervals to examine the phosphorylation level of Sld2. Phosphorylation of a specific amino-acid residue was monitored by Western blotting with indicated anti-phospho antibodies. The phosphorylation level was measured using NIH image and normalized to the level at 120 min as 100%. **(B)** The Sld2-P1 protein (2 pmol) was incubated with the indicated activity of rCdc28-Clb5 at 25°C for 15 min. The phosphorylation level was measured as described in (A) and normalized to the level with 36 units of rCdc28-Clb5 as 100%.

rCdc28-Clb5. Thus, these CDK phosphorylations render Thr84 accessible to DNA-PK. We therefore conclude that phosphorylations other than that to Thr84 render Thr84 accessible to proteins, such as CDK.

Discussion

We showed here that multiple phosphorylations of Sld2 regulate a single specific phosphorylation at Thr84. In eukaryotic cells, multisite phosphorylation in a protein is often observed and this contributes to the regulation of various events, such as multisite docking interactions, integration of different kinase pathways, substrate dephosphorylation, subcellular localization and protein activity (Cohen, 2000). Recently, it was proposed that multisite phosphorylation sets a threshold in a regulated interaction between a CDK inhibitor, Sic1 and the Cdc4 phospho-degron in budding yeast. Sic1 is multiply phosphorylated by CDK and degraded

Figure 6 Phosphorylations other than Thr84 render it accessible to DNA-PK. (A) YK3 (Δ *sld2* (YEp195SLD2)) (Kamimura *et al*, 1998) cells were transformed with one of YCplac22 (Vector), YCpSLD2 (WT) and YCpSLD2P85Q (P85Q). The resulting transformants were streaked onto an FOA plate. (B) Y799 (*drc1-1*; a temperature-sensitive allele of *SLD2*) (Wang and Elledge, 1999) cells harbouring YCp22sld2P85Q were arrested at the G1 phase, released at 36°C and the DNA content was measured as described in the legend to Figure 3D. The DNA content of WT strain (Figure 3D) is also shown as a control. (C) Schematic representation of phosphorylation in the Sld2-P1 protein with a P85Q mutation. (D) The Sld2-P1 protein (12 pmol) with indicated substitution was first incubated with or without rCdc28-Clb5 (4 units) at 30°C for 1 h. The same reactions were incubated in the presence of 10 μ M CDK inhibitor purvalanol A (Gray *et al*, 1998) with or without DNA-PK (25 U: Promega) for further 1 h. Note that DNA-PK alone could not phosphorylate Thr84 of WT Sld2-P1 (Supplementary Figure 4). The asterisk indicates the slower migrating form of the protein.

in a phosphorylation and Cdc4-dependent manner. The multiple suboptimal phosphopeptide motifs in Sic1 for the Cdc4 phospho-degron act in concert to mediate Cdc4 binding and to establish a phosphorylation threshold for Sic1 degradation (Nash *et al*, 2001). In the case of Sld2, multiple phosphorylation sites other than Thr84 do not interact with Dpb11 directly, but rather contribute to the accessibility of a specific phosphorylation motif to CDK. Moreover, as several disruptions at CDK-phosphorylation motifs affect the interaction with Dpb11 through phosphorylation of Thr84 (Figure 4A), at least some CDK phosphorylations in Sld2 seem to work together. This is consistent with the phosphorylation kinetics of Thr84 *in vitro* (Figure 5). We therefore argue that phos-

Figure 7 Regulatory model of the interaction between Dpb11 and Sld2 phosphorylated by CDK. (A) The phosphorylation level of Sld2 is proportional to the level of CDK activity. However, phosphorylation of Thr84 in Sld2 requires prior phosphorylation of other CDK-phosphorylation motifs. When CDK activity increases beyond threshold, Sld2 may change its conformation by multiple phosphorylations and then CDK phosphorylates Thr84. When Thr84 is phosphorylated, Sld2 forms a complex with Dpb11 to initiate DNA replication. (B) When CDK activity increases, the pre-RC components and some other proteins are phosphorylated before Thr84 of Sld2 is phosphorylated. Thus, inactivation of the pre-RC formation and preceding origin association of some replication proteins are ensured.

phorylations of CDK sites work in concert for phosphorylation of Thr84 and thus multisite phosphorylation sets a high threshold of CDK activity for the phosphorylation of Thr84 and consequently for the interaction between Sld2 and Dpb11 (Figure 7A).

This threshold may serve to turn on reactions with pre-determined timing. In the initiation step of DNA replication, the threshold of CDK activity may prevent premature replication in two ways. First, although the activity of G1-phase-specific CDK might not be as high as S-CDK *in vivo*, recombinant Cln2-Cdc28, the yeast G1-CDK, phosphorylated Sld2 *in vitro* as efficiently as did rCdc28-Clb5 (our unpublished result). Thus, the threshold may prevent G1-CDK from causing precocious complex formation between Sld2 and Dpb11. Second, the Sld2-Dpb11 complex does not form until CDK activity increases beyond a threshold, even when S-CDK is activated. This may allow other S-CDK-dependent events to precede Sld2-Dpb11 complex formation. In budding yeast, the pre-RC is formed only in the absence of high CDK activity and its formation is inhibited by CDK-dependent inactivation of the pre-RC components, Orc, Cdc6, Cdt1 and Mcm (Nguyen *et al*, 2001; Tanaka and Diffley, 2002). This threshold ensures phosphorylation of these components before complex formation occurs between Sld2 and Dpb11. Furthermore, many replication proteins assemble on replication origins when CDK activity increases. At this point, some proteins might associate with the origins in a CDK-dependent manner

before any association of the Sld2–Dpb11 complex (Figure 7B). This order might be important, especially at early-firing origins, to initiate DNA replication properly. Possible candidates of these proteins are Sld3 and Cdc45, because they associate with early-firing origins even during the G1 phase and the origin association of Cdc45 is tightened (engaged) in a CDK-dependent manner (Kamimura *et al*, 2001).

CDK activity may regulate multiple reactions to initiate chromosomal DNA replication. Thus, if one reaction is deregulated, the initiation might be perturbed but still regulated by CDK activity. This may be true for the interaction between Sld2 and Dpb11. We constructed an aspartate substitution at Thr84 (T84D) as a phosphomimetic mutation in this study. Although the interaction between the T84D construct and Dpb11 was hardly detected in a protein–protein binding assay (our unpublished result), the two-hybrid assay showed an efficient interaction similar to that with the WT (Figure 4A). We speculate that the WT construct interacts only from the late G1 to early M phases where CDK activity remains high, while the T84D construct interacts with Dpb11 weakly throughout the cell cycle. However, the T84D mutation neither affected cell growth drastically nor caused the cells to initiate chromosomal DNA replication substantially without CDK activity (Figure 3C and our unpublished data), suggesting that a consistent weakly interaction between Sld2 and Dpb11 alone cannot fire the initiation of DNA replication. Thus, in T84D mutant cells, unknown reactions catalysed by CDK may still regulate DNA replication to initiate in a CDK-dependent manner, although the initiation of DNA replication may change in an undetectable level in normal conditions.

Although we do not know exactly how phosphorylations of the sites other than Thr84 render Thr84 accessible to CDK, we can explain it in two ways. First, phosphorylations change a conformation on Sld2. The Thr84 site is hindered from binding to CDK, and phosphorylations of the sites other than Thr84 change a conformation on Sld2 so that Thr84 is released to be accessible to CDK (Figure 7A). Second, to phosphorylate Thr84, CDK requires interactions with prior phosphorylation sites. Thus, phosphorylations at other sites enhance phosphorylation at Thr84 through the interaction between CDK and phospho-residues of other sites in Sld2. We prefer the model of a conformational change because phosphorylation of Thr84 by DNA-PK in a P85Q construct still required CDK-catalysed phosphorylations (Figure 6D). As CDK and DNA-PK differ significantly, we do not think that both kinases function through the interaction with phospho-residues in Sld2. Thus, a conformational change is a more plausible explanation than the interaction between a kinase and phospho-residues. Moreover, although we do not know the structure of Sld2, phosphorylated and unphosphorylated Sld2 showed slightly different digestion patterns with proteases (our unpublished result). This result further supports a conformational change caused by phosphorylation.

When Thr84 is phosphorylated by CDK ultimately, the short stretch containing pThr84 binds to Dpb11. However, when we substituted the PIKK phosphorylation motif Thr–Gln for CDK-phosphorylation motif Thr–Pro, the resulting mutant (P85Q) was replaceable for WT (Figure 6A). In *mec1-kd sml1Δ* background, in which Mec1 kinase activity is defective and lack of *sml1* restores growth defect of *mec1-kd* (Takata *et al*, 2004), the P85Q mutant showed slower

growth than the WT *SLD2* (our unpublished result). This observation suggests that at least Mec1, one of PIKKs, is partially responsible for Thr84 phosphorylation. Although Mec1-dependent phosphorylations are observed when DNA is damaged or replication forks are stalled, Mec1 also phosphorylates the single-stranded DNA-binding protein, replication protein A (RPA), in the S and G2 phases during normal cell cycle progression (Brush *et al*, 1996). We thus surmise that Thr84 in the P85Q construct of Sld2 is phosphorylated by PIKKs during normal cell cycle progression.

The predicted binding domains of Sld2 homologues are accompanied by multiple CDK-phosphorylation motifs (Figure 1A). Thus, the regulatory mechanism of Thr84 phosphorylation in Sld2 seems to be generally conserved in yeast and fungi homologues. RECQL4 has been proposed as a homologue of Sld2 in vertebrates because its N-terminal 200-amino-acid region has homology to Sld2 and it is essential for DNA replication in *Xenopus* egg extracts (Sangrithi *et al*, 2005). *Xenopus* RECQL4 encodes a 1500-residue protein with the 380-amino-acid helicase domain and the 500-amino-acid region bearing a cluster of CDK-phosphorylation motifs (Supplementary Figure 1A). Although its association with chromatin does not require CDK activity, it is conceivable that the RECQL4 function is regulated by CDK-catalysed multiple phosphorylation. We thus suggest that the mechanism proposed in this study governs CDK-catalysed multiple phosphorylations of Sld2 and its homologues to regulate the initiation of DNA replication. Moreover, this mechanism may take place in CDK-dependent complex formations other than Sld2 family proteins because CDK-phosphorylation motifs are clustered even in CtIP and BACH1, which bind to a pair of the BRCT domains of BRCA1 when their motifs in a binding stretch are phosphorylated (Supplementary Figure 1B) (Yu *et al*, 2003; Yu and Chen, 2004).

Materials and methods

Preparation of Sld2 and Dpb11 proteins

Sld2-P1 (residues 79–263) tagged with Flag on pT7-FLAG1 (Sigma Aldrich) and Dpb11-C (residues 291–631) fused to GST on pGEX6P-1 (Amersham Bioscience) were expressed in *E. coli* BL21 CodonPlus (Stratagene) at 25°C. Sld2-P1 in bacterial lysates was bound to anti-FLAG M2 affinity Gel (Sigma Aldrich), eluted with 3 mg/ml 3 × Flag peptide (Sigma Aldrich) in 20 mM Tris–Cl buffer (pH 7.5) and further purified by Resource S column chromatography (Amersham Biosciences). Dpb11-C was retained on glutathione Sepharose 4B, eluted with 20 mM glutathione/50 mM Tris–Cl (pH 8.0) and further purified by Mono Q column chromatography.

Phospho-Ser- or -Thr-specific antibodies

Phospho-specific rabbit antibodies were raised against KLH-conjugated peptides, CDEVVEIGPpTPQVYVK for anti-pThr84, CSIFDMNLpSPIKPIY for anti-pSer100 and CSGYYGNpSPLKLDE for anti-pSer208. Antibodies were purified using specific phosphorylated peptide-conjugated columns and adsorbed against the corresponding unphosphorylated peptide. The antibodies were detected with horseradish peroxidase-conjugated swine anti-rabbit or goat anti-mouse antibodies (Bio Rad) and enhanced chemiluminescence substrates (Amersham Biosciences), or with either Alexa Fluor 680 Goat Anti-rabbit IgG (Molecular Probes) or IRDyeTM 800CW Conjugated Affinity Purified Anti-Mouse IgM (Rockland) using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

Preparation of yeast CDKs

To obtain an active recombinant Cdc28/Clb5 complex (rCdc28–Clb5), we constructed the pGEX6P-1/CDC28–CAK1–CKS1–CLB5 plasmid consisting of multiple open reading frames encoding the

GST-fused Cdc28 kinase, its activating Cak1 kinase, Cks1 subunit and Clb5 S-phase cyclin (Mendenhall and Hodge, 1998) in one polycistronic unit similarly as described (Ito and Kurosawa, 1992). The cell lysate prepared from *E. coli* BL21-CodonPlus(DE3)-RIL cells harbouring the plasmid was applied to glutathione (GSH)-Sepharose (Amersham Biosciences) and the retained fractions were further purified by Resource Q (Amersham Biosciences) column chromatography. To remove GST from Cdc28 fusion protein in the CDK complex, samples were bound to GSH-Sepharose and then mixed with PreScission protease (Amersham Biosciences). Then, the released fractions were subjected to Phenyl-5PW (Toso) column chromatography and active fractions were stored. One unit of rCdc28-Clb5 was defined as the activity that rendered 1 pmol unphosphorylated Sld2-P1 to its slowest migrating form in SDS-PAGE at 30°C for 1 h.

Phosphorylation of Sld2

Phosphorylation of Sld2 was performed in 50 mM Na β -glycerophosphate, 15 mM Mg-acetate, 13 mM EGTA, 1 mM DTT and 0.3 mM ATP, containing FLAG-Sld2-P1 protein at 0.2–5 μ M and an aliquot of rCdc28-Clb5. For kinetic assays, reactions were incubated at 25°C and stopped by adding 2% SDS-loading buffer, followed by incubation at 100°C for 3 min. Phosphorylated substrates were resolved in 4–20% gradient SDS-PAGE and analysed by Western blotting. Using both phospho-peptides and serial dilutions of phosphorylated Sld2-P1, we confirmed that signal strengths in Western blotting reflected phosphorylation levels accurately.

Protein–protein binding assay

Biotinylated peptides (250 pmol) were mixed with 20 μ l of Dynabeads M-280 streptavidin (Dyna) in 50 μ l of B2 buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.5% NP-40) at 4°C for 1 h and then further incubated with 0.2 μ g of biotin for

30 min to reduce nonspecific binding. GST-Dpb11-C, which had been diluted with 10 volumes of B2 containing 5 mg/ml BSA was incubated with peptide beads at 4°C for 30 min. The beads were washed with 100 μ l of B2 containing 0.5 M NaCl three times and then eluted with 20 μ l of SDS sample buffer. As purified Dpb11 proteins tend to bind to resins nonspecifically, we always added BSA. Therefore, we could not measure the K_d values of Sld2 and Dpb11.

For phosphorylated or unphosphorylated Flag-Sld2 P1, GST-Dpb11-C immobilized on GSH-Sepharose (Amersham Biosciences) beads was mixed with Sld2 in 50 μ l of buffer B (20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM EDTA, 0.5% Triton X-100, protease inhibitor complete (Roche), phosphatase inhibitor cocktail (Sigma Aldrich)) containing 3 mg/ml BSA, incubated at 4°C for 1 h and then washed three times with 100 μ l of B buffer containing 0.5 M NaCl. For competition assays, various peptides and phosphorylated Flag-Sld2 were incubated with immobilized GST-Dpb11-C at 4°C for 1 h. The proteins retained on beads were analysed by SDS-PAGE and Western blotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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