p75 (a surface receptor) served as a cell-surface control for siRNA-induced PLC-γ1 depletion.

Reagents

Enhanced yellow fluorescent protein (YFP) vector and Lipofectamine were from Clontech; anti-Myc, [^{35}S]methionine, carbachol, ONPG and GST–Sepharose were from Sigma; Fura-2/acetoxymethyl ester was from Molecular Probes. siRNA duplexes were from Dharmacon Research. Anti-p75 antibody was from Upstate.

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Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates

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Cell-cycle events are controlled by cyclin-dependent kinases (CDKs), whose periodic activation is driven by cyclins. Different cyclins promote distinct cell-cycle events, but the molecular basis for these differences remains unclear[1,2]. Here we compare the specificity of two budding yeast cyclins, the S-phase cyclin Clb5 and the M-phase cyclin Clb2, in the phosphorylation of 150 Cdk1 (Cdc28) substrates. About 24% of these proteins were phosphorylated more efficiently by Clb5–Cdk1 than Clb2–Cdk1. The Clb5-specific targets include several proteins (Sld2, Cdc6, Orc6, Mcm3 and Cdh1) involved in early S-phase events. Clb5 specificity depended on an interaction between a hydrophobic patch in Clb5 and a short sequence in the substrate (the RXL or Cy motif). Phosphorylation of Clb5-specific targets during S phase was reduced by replacing Clb5 with Clb2 or by mutating the substrate RXL motif, confirming the importance of Clb5 specificity *in vivo*. Although we did not identify any highly Clb2-specific substrates, we found that Clb2–Cdk1 possessed higher intrinsic kinase activity than Clb5–Cdk1, enabling efficient phosphorylation of a broad range of mitotic Cdk1 targets. Thus, Clb5 and Clb2 use distinct mechanisms to enhance the phosphorylation of S-phase and M-phase substrates.

A long-standing question in cell-cycle control is how different cyclins drive the distinct events of S phase and M phase[1,2]. One model, termed the quantitative model of cyclin function, suggests that S phase is triggered by low levels of cyclin–CDK activity and M phase is initiated at higher levels of activity[3,4]. According to this model, apparent differences in cyclin function are due primarily to differences in their timing and levels of expression. In contrast, the qualitative model proposes that different cyclins possess different intrinsic functional capacities, perhaps because they modulate the substrate specificity of the associated CDK or alter its subcellular location[1,5]. Studies in budding yeast, for example, argue that the S-phase cyclin Clb5 possesses higher intrinsic S-phase-promoting activity than the M-phase cyclin Clb2 (refs 6, 7). Biochemical studies with mammalian cyclins have demonstrated cyclin specificity in the phosphorylation of a small number of substrates: for example, mammalian cyclin-A–CDK, but not cyclin-B–CDK, phosphorylates the pRb-related protein p107 (ref. 8). However, few cyclin-specific subtrates have been identified in any species, and the general importance of cyclins in CDK substrate specificity remains unclear.

To assess the global importance of cyclin specificity in cell-cycle control, we measured the phosphorylation of a large number of Cdk1 substrates by S-phase and M-phase cyclin–CDK complexes. We recently identified 181 budding yeast Cdk1 substrates[9], and in the present study we measured the kinase activities of Clb5–Cdk1 and Clb2–Cdk1 towards 150 of these proteins (obtained from proteomic libraries; see Methods). To prevent background phosphorylation by contaminating protein kinases in the reactions, we used a mutant form of Cdk1, Cdk1-as1, that contains an enlarged ATP-binding site. Purified Clb5–Cdk1-as1 or Clb2–Cdk1-as1 complexes were incubated with the test substrate and the bulky ATP analogue [γ-^{32}P]N^6-(benzyl)ATP, which only the mutant Cdk1-as1 enzyme can use[9]. Reactions were performed with amounts of Clb5–Cdk1-as1 and Clb2–Cdk1-as1 that possessed equal activities toward the non-specific substrate histone H1 (Fig. 1a). Phosphate incorporation was then divided by the amount of substrate protein, and the logarithm of this ratio was designated as the substrate P-score, as described previously[9]. All reactions were performed at very low substrate concentrations (presumably well below K_m); thus, differences in P-scores between the two kinases provide a reasonable estimate of relative k_{cat}/K_m values, where K_{cat} is catalytic constant and K_m is Michaelis constant.

All substrate P-scores for the two kinases are plotted in Fig. 1b. These data suggest that, on a histone H1-normalized scale, most of the substrates – about 110 of the 150 – are equally good substrates for Clb5–Cdk1 and Clb2–Cdk1, because they exhibit 2.5-fold or less specificity for either kinase and fall in the middle diagonal region of the plot. Most of the remaining substrates, falling to the right of the diagonal, are specific for Clb5. Among these were 14 substrates with specificity for Clb5 ranging from 10-fold to 800-fold, whereas 22 proteins displayed specificity of between 2.5-fold and 10-fold (note that the scale on this plot is logarithmic). The top Clb5-specific substrates included several proteins involved in DNA replication (Orc6, Orc2, Mcm3, Cdc6 and Sld2), spindle pole body function

(Mps2 and Spc110), APC activation (Cdh1) and other functions (Supplementary Table 1). Surprisingly, we found no highly Clb2-specific substrates and only a few substrates that displayed minimal (2.5–3.0-fold) specificity for Clb2.

We next determined the mechanism underlying Clb5 specificity. Substrate recognition by cyclin–CDK complexes is known to be governed primarily by an interaction between the substrate's consensus phosphorylation sequence, S/T*PXK/R, and the CDK active site[10]. In addition, a region on the surface of some cyclins, called the hydrophobic patch, has been reported to interact with a sequence motif called an RXL or Cy motif on some CDK substrates and inhibitors[10–14]. Mutation of the hydrophobic patch decreases the biological activity, but not the histone H1 kinase activity, of the Clb5–Cdk1 complex[15], but the broad significance of this interaction for CDK substrate targeting remains unclear. We therefore analysed the effects of hydrophobic patch mutations on the phosphorylation of Clb5-specific substrates identified in our screen. We found that the high Clb5 specificity of these substrates was entirely dependent on a functional hydrophobic patch (Fig. 2a). Mutation of the hydrophobic patch in Clb2 had no significant effect on its activity towards these substrates, and patch mutations in either cyclin had no effect on the phosphorylation of non-specific substrates such as histone H1 (Fig. 2a).

More detailed kinetic analyses were performed with two Clb5-specific substrates, the replication protein Cdc6 and the putative spindle protein Fin1 (ref. 16), which were chosen because they could be prepared in the large amounts needed for these studies. In both cases, the hydrophobic patch-dependent substrate interaction resulted in highly efficient phosphorylation, with K_m values in the low micromolar range (Fig. 2b).

We also attempted to identify substrate regions that interact with the hydrophobic patch of Clb5. With the use of site-directed mutagenesis, we screened potential RXL, RXF or KXL motifs in the Clb5-specific substrate Fin1 and found that a single KXL motif (residues 191–193) was almost entirely responsible for the high Clb5 specificity (Fig. 2c). Mutation of this sequence resulted in a 30-fold decrease in k_{cat}/K_m with Clb5–Cdk1 (data not shown). The rate of Fin1 phosphorylation by Clb2–Cdk1 was not significantly affected by mutation of this motif (Fig. 2c) or any of the other related motifs in Fin1 (data not shown).

During the course of these studies, we noticed that purified Clb5–Cdk1 had a lower histone H1 kinase activity than Clb2–Cdk1. This difference was not due to differences in binding of the CDK inhibitor Sic1, because the kinase complexes were purified from a strain lacking Sic1. To address this issue further, we performed kinetic analyses with a peptide substrate that is derived from histone H1 and is ideal for characterization of the CDK active site without interference from other interactions. Purified Clb2–Cdk1 was about 10–20-fold more active towards this peptide or histone H1 than purified Clb5–Cdk1 (Table 1). The same was true for the mutant Cdk1-as1 complexes used for the proteomic screen described above (data not shown). The difference in activities was due almost entirely to a tenfold difference in K_m values for the peptide substrate (Table 1), whereas k_{cat} and K_m values for ATP (data not shown) were similar for both enzymes. A similar difference in peptide K_m was observed with Clb5- and Clb2-associated kinases immunoprecipitated from cell lysates (data not shown). These results, combined with the similarity in k_{cat} values for the two complexes, argue that the difference in activities of the two kinases was not due to partial inactivation of Clb5–Cdk1 during purification.

These data reveal a previously unrecorded principle of cyclin function: rather than simply activating a CDK, different cyclins can differentially modulate the intrinsic properties of the CDK active site. These data also suggest that, with the exception of specific substrates that interact with the Clb5 hydrophobic patch, Clb5–Cdk1 has lower activity than Clb2–Cdk1 towards the general substrates that lie along the diagonal of Fig. 1b. Thus, if the data in Fig. 1b had been obtained with equal kinase protein amounts rather than equal histone H1 kinase activities, P-scores for Clb2 would have increased by more than 1 unit, resulting in a majority of proteins that were above the diagonal and could therefore be considered Clb2-specific.

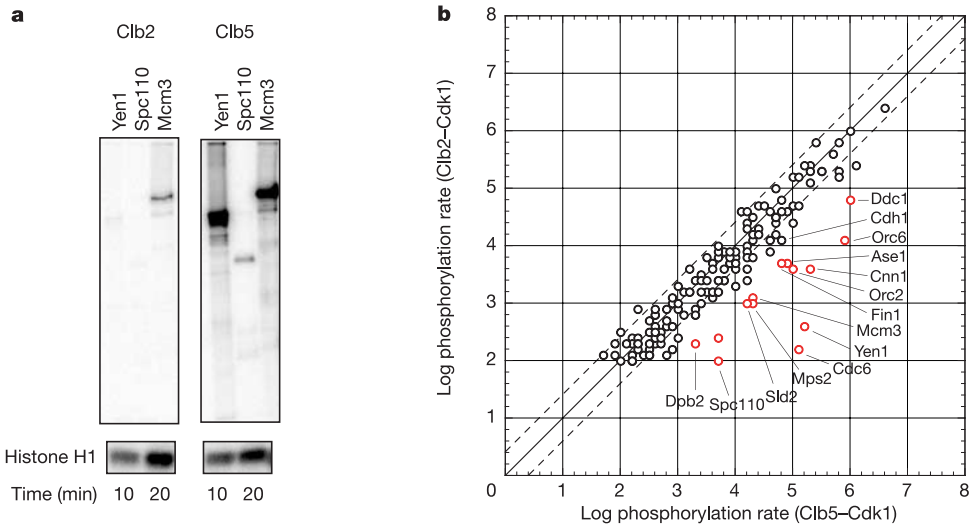

Figure 1 Identification of Clb5-specific Cdk1 substrates in budding yeast. **a**, Representative data showing phosphorylation of three Clb5-specific substrates. Purified Clb2–Cdk1-as1 (left) and Clb5–Cdk1-as1 (right) were normalized for histone H1 kinase activity (lower panels) and incubated with the indicated GST-tagged substrate proteins and [γ-^{32}P]N^6-(benzyl)ATP. Reaction products were analysed by SDS–PAGE and autoradiography. **b**, Rates of phosphorylation of 150 substrates by Clb2–Cdk1 and Clb5–Cdk1 were divided by substrate amount and are plotted here as logarithmic values. The activities of the two kinases were normalized to histone H1 kinase activity. The central diagonal region (bounded by dashed lines) contains substrates whose phosphorylation rates with the two kinases were similar (less than a 2.5-fold difference in phosphorylation rate). Red circles indicate 14 substrates that are phosphorylated more than tenfold more rapidly by Clb5–Cdk1 than by Clb2–Cdk1. Phosphorylation rates for all substrates are given in Supplementary Table 1.

Finally, to investigate the effects of cyclin specificity *in vivo*, we analysed the phosphorylation state of several Cdk1 substrates during the cell cycle in yeast strains in which the open reading frame of *CLB5* was replaced with that of *CLB2* at the *CLB5* locus. As shown previously[6], the *clb5::CLB2* strain provides an excellent system in which to compare the intrinsic specificity of the two cyclins in the absence of normal differences in the timing of cyclin expression. Clb2 expression in the *clb5::CLB2* strain increased at the same time in the cell cycle as Clb5 in a wild-type strain (Fig. 3a). Clb2-associated histone H1 kinase activity in immunoprecipitates from the *clb5::CLB2* strain was about 3–4-fold greater than that of Clb5 from a wild-type strain (Fig. 3b). This difference is less than that seen with purified kinases, presumably due to relatively low Clb2 expression from the *CLB5* locus (Fig. 3a).

We first analysed the phosphorylation of the Clb5-specific substrate Fin1 after release from G1 arrest. Fin1 displays a Cdk1-dependent mobility shift in western blots[9]. Here we found that Fin1 in wild-type cells was fully phosphorylated immediately after its synthesis in early S phase, and was then dephosphorylated after degradation of Clb5 in mitosis (despite the continued presence of Clb2; Fig. 3c). However, in the *clb5::CLB2* strain, Fin1 phosphorylation during S phase was decreased by 30–40%. Similar results were obtained when Fin1 was replaced by a form in which the KXL motif was mutated. A decrease in phosphorylation of this magnitude is likely to reflect a large decrease in the rate of phosphorylation *in vivo*, as argued by theoretical considerations of the effects of changing kinase and phosphatase activities on substrate phosphorylation state[17]. For example, if the kinase and phosphatase acting on a substrate are not saturated with substrate (that is, under first-order conditions), then a 99-fold decrease in kinase activity is required for a decrease in substrate phosphorylation from 99% to 50%. Our data therefore indicate that Fin1 is a highly Clb5-specific substrate *in vivo* and that the interaction between the KXL motif of Fin1 and the hydrophobic patch of Clb5 is important for this specificity.

Table 1 **Kinase activities of Clb2–Cdk1 and Clb5–Cdk1**

Kinase	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} min^{-1}$)
Clb2–Cdk1	Histone peptide	45.9 ± 15.4	189 ± 14	4.1
	Histone H1	–	–	4.9
	Fin1	–	–	6.4
Clb5–Cdk1	Histone peptide	521 ± 98	114 ± 9	0.22
	Histone H1	–	–	0.63
	Fin1	3.1 ± 0.5	102 ± 16	33.0

Kinetic properties of purified Clb2–Cdk1 and Clb5–Cdk1 with a histone H1-derived substrate peptide (PKTPKKAKKL) were compared with activity towards histone H1 and the Clb5-specific substrate Fin1. Virtually identical results were obtained with another commonly used peptide substrate (ADAQHATPPKKKRKVEDPKDF; data not shown). Values are means ± s.d.

Similar results were obtained with the Clb5-specific substrate Sld2 (Fig. 3d). Replacement of Clb5 by Clb2 delayed the onset and decreased the amount of Sld2 phosphorylation, helping to explain previous evidence that the intrinsic S-phase-promoting activity of Clb2 in this strain is less than that of Clb5 (ref. 6). We also analysed a protein, Slk19, that exhibits no apparent specificity for Clb5 or Clb2 *in vitro*. Patterns of Slk19 phosphorylation were similar in wild-type and *clb5::CLB2* strains after release from G1 arrest (Fig. 3e), further indicating that the cyclin specificities observed *in vitro* (Fig. 1b) are relevant *in vivo*.

Thus, our large-scale comparative analysis of Cdk1 specificity unveiled a large group of highly specific protein targets for the S-phase cyclin Clb5. At the onset of S phase, this specificity might contribute to the switch-like phosphorylation of substrates required for efficient S-phase progression. Phosphorylation of Sld2, for example, is needed for the initiation of replication[18], Cdh1 phosphorylation allows the accumulation of cyclins and other regulators[19], and proteins of the pre-replicative complex (Cdc6, Orc2,

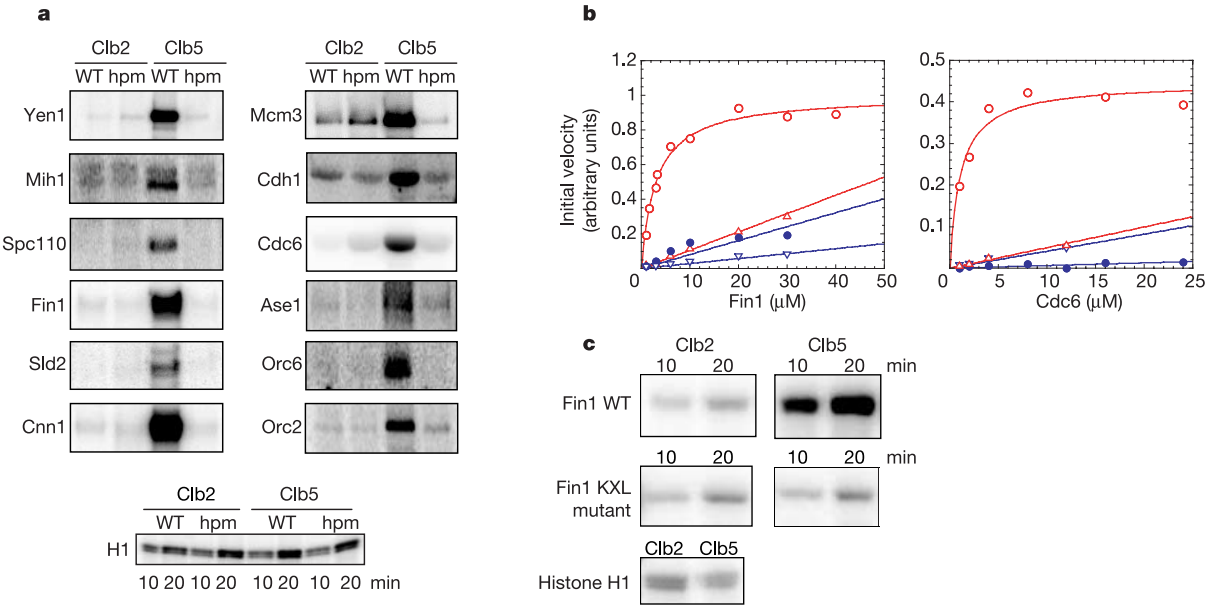

Figure 2 Clb5 specificity depends on an interaction between the Clb5 hydrophobic patch and an RXL motif in the substrate. **a**, Effect of the hydrophobic patch mutation (hpm) on the rate of Clb2–Cdk1- and Clb5–Cdk1-catalysed phosphorylation of substrates. Selected substrates with a high degree of Clb5 specificity were phosphorylated by purified Clb2–Cdk1, Clb2hpm-Cdk1, Clb5–Cdk1 and Clb5hpm-Cdk1. As a control, histone H1 phosphorylation is also shown at two time points (bottom panel). WT, wild type. **b**, Steady-state kinetic characterization of Clb5-specific substrates Fin1 (left) and Cdc6 (right), using purified Clb2–Cdk1 (blue circles), Clb2hpm-Cdk1 (blue triangles), Clb5–Cdk1 (red circles) and Clb5hpm-Cdk1 (red triangles) (normalized for histone H1 kinase activity). For Fin1, $K_{m,Clb5} = 3.1$ μM; for Cdc6, $K_{m,Clb5} = 1.1$ μM. **c**, A KXL motif in Fin1 is responsible for the Clb5 specificity of Fin1 phosphorylation. A standard kinase assay, normalized for histone H1 kinase activity, was performed with wild-type Fin1 and with a KXL mutant in which residues Lys 191 and Leu 193 were changed to alanine.

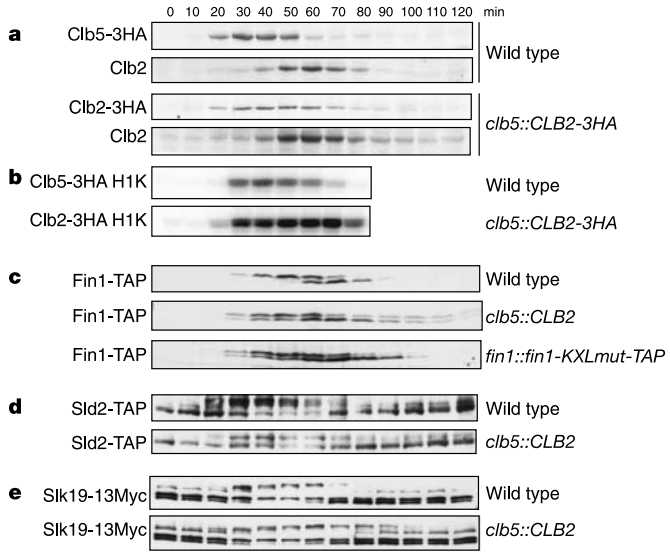

Figure 3 Clb5-specific substrate phosphorylation occurs *in vivo*. The indicated yeast strains were arrested in G1 with 1 μg ml^{-1} α-factor and then released from the arrest. New α-factor was added after the initiation of budding, to prevent entry into the next cell cycle. Cells were harvested for western blotting or kinase assays at the indicated times. **a**, western blotting of the indicated cyclins in lysates from a wild-type strain carrying *CLB5-3HA* at the *CLB5* locus, or a strain carrying *CLB2-3HA* at the *CLB5* locus (*clb5::CLB2-3HA*). **b**, Histone H1 kinase activity was measured in immunoprecipitates of Clb5-3HA from the wild-type strain and Clb2-3HA from the *clb5::CLB2-3HA* strain. **c**, **d**, Phosphorylation-dependent mobility shifts were measured by western blotting of the Clb5-specific substrates Fin1 (**c**) and Sld2 (**d**) (each TAP-tagged) in wild-type and *clb5::CLB2* strains. In both cases the upper band represents the phosphorylated form. A similar experiment was performed in a strain carrying the *fin1-KXL* mutation at the *FIN1* locus. Quantification of the two Fin1 bands at the 30–50-min time points revealed that Fin1 is 100% phosphorylated in wild-type cells, 63–68% phosphorylated in *clb5::CLB2* cells and 59–63% phosphorylated in *fin1-KXLmut* cells. **e**, Similar studies were performed with 13Myc-tagged Slk19, an example of a protein with no significant cyclin specificity. Slk19 migrates as two major bands, the lower of which is a proteolytic fragment produced by separase. Each of the two bands displays decreased mobility after phosphorylation[9].

Orc6 and Mcm3) must be phosphorylated to prevent DNA re-replication[20]. Consistent with this is the recent observation[14] that Clb5 binding to an RXL motif in Orc6 is important for the suppression of DNA re-replication. Clb5 specificity might also be important in late mitosis. Clb5 is degraded in anaphase, before most Clb2 (Fig. 3a)[21,22], and it can be imagined that early dephosphorylation of Clb5-specific substrates might contribute in some way to the control of late mitotic events.

Our work also revealed that Clb2–Cdk1 has higher kinase activity than Clb5–Cdk1. This difference is due almost entirely to a tenfold difference in K_m values for general substrates (Table 1). K_m differences of this magnitude will have important consequences inside the cell, where these kinases are exposed to high substrate concentrations. The concentrations of Cdk1 substrates in budding yeast are difficult to estimate, because some substrates are likely to be localized to specific subcellular sites. Nevertheless, a rough estimate can be made on the basis of recent proteomic studies[23,24]. If we combine the concentrations of the 181 CDK substrates we identified[9], then a conservative estimate of the total concentration of CDK phosphorylation sites in the nucleus would be at least 1.0 mM. This concentration greatly exceeds the relatively low substrate K_m values (about 50 μM) of Clb2–Cdk1 for general substrates. The saturation of Clb2–Cdk1 by these substrates should initially reduce the rate of phosphorylation of general substrates not involved in early S-phase events. This initial suppression of Clb2–Cdk1 activity probably explains our observation (Fig. 3e) that phosphorylation of the general substrate Slk19 in wild-type and *clb5::CLB2* cells occurs gradually during progression through S phase, despite the fact that Slk19 is a highly efficient substrate *in vitro*. As Clb2 concentrations increase and general substrates become phosphorylated, inhibition by unphosphorylated substrates will eventually be relieved, allowing Clb2–Cdk1 to assume its role as a highly efficient kinase for the large number of mitotic substrates. In contrast, the relatively inefficient Clb5–Cdk1, because of its high K_m values for general substrates (about 500 μM), might not be significantly inhibited by these proteins. This lower degree of competition from general substrates allows Clb5–Cdk1 to focus, through interactions between the hydrophobic patch and the RXL motif, on a subset of low K_m substrates whose phosphorylation is critical for S-phase initiation. The low activity of Clb5–Cdk1 towards general targets, combined with its high affinity for specific targets (see also refs 14, 25), might explain the observation that overexpressed Clb5 is unable to replace Clb2 function in blocking mitotic exit[26].

Our studies therefore support the existence of multiple mechanisms by which the intrinsic biochemical properties of different cyclins help to promote the correct timing of CDK substrate phosphorylation during the cell cycle. We argue that there is a fundamental advantage of this specificity over the quantitative model of cyclin function. In the quantitative model, M-phase CDK targets must have very low specificity for the kinase, because S-phase Cdk1 activity would otherwise cause their partial phosphorylation and the two phases would not be well separated. These weak substrates would then require extremely high mitotic cyclin–CDK activity to achieve significant levels of phosphorylation in M phase. However, a system employing cyclins with specific functional capacities can operate with cyclin concentrations that are lower and change less markedly, resulting in a far more efficient and robust mechanism by which the events of S and M phases are triggered in the correct order and do not overlap. □

Methods

General methods

All strains were derivatives of W303 or S288C and were grown at 30 °C. Construction of epitope-tagged strains was performed as described[27]. To construct hydrophobic patch mutations, three residues in Clb5 (Met 197, Leu 201 and Trp 204) and Clb2 (Asn 260, Leu 264 and Trp 267) were changed to alanine[15]. Protein extracts were prepared for immunoblotting as described[9]; tandem affinity purification (TAP) and 13Myc tags were detected by c-Myc polyclonal antibody (Santa Cruz); the three-haemagglutinin (3HA) tag was detected with the 16B12 antibody (Covance), and endogenous Clb2 was detected with polyclonal anti-Clb2 antibody (a gift from D. Kellogg). Band intensities on immunoblots were quantified by the analysis of scanned films with ImageQuant software.

Kinase purification

CDK–cyclin complexes (Cdk1–Clb2-TAP, Cdk1–Clb5-TAP, Cdk1-as1–Clb2-TAP, Cdk1-as1–Clb5-TAP, and the corresponding versions with hydrophobic patch mutations) were purified to homogeneity as described[9,28] from yeast strains lacking *SIC1* and expressing the desired TAP-tagged cyclin under the control of the *GAL* promoter. Fin1 and Cdc6 were expressed in bacteria as His_6-tagged proteins (in pET28b) and purified by metal-affinity chromatography on a cobalt–IDA (iminodiacetic acid) column.

Kinase assays

Comparison of substrate phosphorylation with the two cyclins was performed with purified Clb–Cdk1-as1 complexes by two methods, as follows. First, glutathione *S*-transferase (GST)-tagged proteins[29] were incubated in total cell extracts with Clb–Cdk1-as1 and [γ-^{32}P]N^6-(benzyl)ATP as described[9]. PhosphorImager units of substrate phosphorylation were divided by substrate amount as determined from silver-stained gels[9]. Second, for substrates that were difficult to detect in the GST-tagged library, TAP-tagged proteins[24] in yeast cell lysates were enriched by immobilization on magnetic IgG-coupled beads and washed; they were then subjected to kinase reactions as described above. In these cases, relative P-scores were obtained and scaled by using previously obtained P-scores for Clb2 (ref. 9). About 20 substrates – including most of the highly Clb5-specific targets – were also tested with wild-type Cdk1–Clb complexes, and the results showed that the analogue-sensitive mutation does not influence the degree of cyclin specificity.

Although our methods provide a valid approach to measuring the specificity of different cyclins for the same substrate, the significance of P-score differences for different substrates with the same cyclin remains uncertain. Substrates along the diagonal in Fig. 1b, for example, are phosphorylated at rates that vary by more than four orders of magnitude.

These differences might result from several factors, including differences in the number of phosphorylation sites on substrates, inaccuracies in our estimates of the amount of substrate in the reaction, and problems with the proteolysis and folding of GST fusion proteins. In addition, the significance of different P-scores for different proteins is difficult to assess without any knowledge of the rate at which these proteins are dephosphorylated in the cell.

Detailed kinetic analyses were performed with [γ-^{32}P]ATP in a reaction mixture containing 25 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 0.1 mM ATP, 2 mM $MgCl_2$ and purified Clb–Cdk1 complex. Apparent K_m values were determined from initial velocities of substrate phosphorylation (up to 10% of total substrate turnover) at different substrate concentrations. Phosphorylation of protein substrates was quantified by PhosphorImager analysis of polyacrylamide gels. For peptide substrate assays, peptides were bound to phosphocellulose paper, washed with 75 mM orthophosphoric acid and quantified by counting Cerenkov radiation. For kinase assays in immunoprecipitates, cells were lysed by bead-beating in RIPA buffer, and Cdk1–Clb-3HA complexes were immunoprecipitated with 16B12 antibody and protein-G-coupled magnetic beads (Dynal Biotech). Conventional kinase assays with [γ-^{32}P]ATP and histone H1 were then performed. Peptide substrates were obtained from Promega and Sigma.

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Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1

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Spinocerebellar ataxia with axonal neuropathy-1 (SCAN1) is a neurodegenerative disease that results from mutation of tyrosyl phosphodiesterase 1 (TDP1)[1]. In lower eukaryotes, Tdp1 removes topoisomerase 1 (top1) peptide from DNA termini during the repair of double-strand breaks created by collision of replication forks with top1 cleavage complexes in proliferating cells[2–4]. Although TDP1 most probably fulfils a similar function in human cells, this role is unlikely to account for the clinical phenotype of SCAN1, which is associated with progressive degeneration of post-mitotic neurons. In addition, this role is redundant in lower eukaryotes, and Tdp1 mutations alone confer little phenotype[4–7]. Moreover, defects in processing or preventing double-strand breaks during DNA replication are most probably associated with increased genetic instability and cancer, phenotypes not observed in SCAN1 (ref. 8). Here we show that in human cells TDP1 is required for repair of chromosomal single-strand breaks arising independently of DNA replication from abortive top1 activity or oxidative stress. We report that TDP1 is sequestered into multi-protein single-strand break repair (SSBR) complexes by direct interaction with DNA ligase IIIα and that these complexes are catalytically inactive in SCAN1 cells. These data identify a defect in SSBR in a neurodegenerative disease, and implicate this process in the maintenance of genetic integrity in post-mitotic neurons.

To investigate the molecular basis of SCAN1, normal and SCAN1 lymphoblastoid cells were compared for levels of DNA breakage during a 1-h treatment with camptothecin (CPT). CPT increases the half-life of cleavage complex intermediates of top1 activity and so increases the likelihood of their conversion into strand breaks[9], which can be measured with the alkaline comet assay[10]. Normal cells accumulated low levels of breakage during the first 20 min of treatment with CPT, which then typically fell to near-background levels by the end of treatment (Fig. 1a). In contrast, SCAN1 cells