Rescue of a Human Cell Line from Endogenous Cdk1 Depletion by Cdk1 Lacking Inhibitory Phosphorylation Sites

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Cells that transiently overexpress cyclin-dependent kinase 1 lacking inhibitory phosphorylation sites (Cdk1-AF) undergo premature and catastrophic mitosis, reflecting the key role for Cdk1 in promoting a timely transit from G2 into mitosis. Conversely, cells depleted of Cdk1 undergo repeated S phases without intervening mitoses (endoreduplication), reflecting a role for Cdk1 in preventing premature S phases. It is not known how Cdk1 prevents entry into S phase at times in G2 when it does not promote mitosis. Also uncertain is the extent of redundancy between inhibitory phosphorylation and other mechanisms for controlling Cdk1 activity. We describe here human cells that not only tolerate stable Cdk1-AF expression but also rely on it for survival when endogenous Cdk1 is depleted. When residual endogenous Cdk1 expression is further depleted, however, proliferation of Cdk1-AF-rescued cells is inhibited. Interestingly, this inhibition is not accompanied by endoreduplication. These results are consistent with a two-threshold model for Cdk1 kinase activity, one for suppressing endoreduplication and one for promoting mitosis. They also indicate that inhibitory phosphorylation is indispensable for only a fraction of the total cellular complement of Cdk1.

Cyclin-dependent kinases (Cdks) control eukaryotic cell cycle progression, ensuring that each phase occurs in an orderly and timely manner (1–3). To be active, Cdks must be bound to an appropriate cyclin molecule and free from inhibition by Cdk inhibitors. Their action can be further controlled by changes in subcellular localization and phosphorylation state. Cdks therefore have a variety of ways in which their activity can be modulated in response to extrinsic (e.g. presence of growth factor) or intrinsic (e.g. completion of DNA synthesis) cues (4–8).

Transitions from G2 to mitosis and from G1 into S phase are key cell cycle control points. Simple eukaryotes have a single Cdk (Cdk1, or Cdc2 and Cdc28 in budding and fission yeast, respectively) that controls both of these transitions. Higher eukaryotes have multiple Cdks (Cdk1 to Cdk11) with more specialized roles, but Cdk1 and its role in promoting mitosis have been conserved. In the normal cell cycle, mitotic cyclins (cyclins A, B, and B3) accumulate during G2, and Cdk1 is held in a relatively inactive state by inhibitory phosphorylation at residues Tyr15 (in fission yeast) or Thr14 and Tyr15 (higher eukaryotes). Two kinases (Myt1 and Wee1) and one phosphatase (Cdc25) determine the phosphorylation status of these residues, and, together with Cdk1, these are thought to constitute a molecular switch that commits cells to undergo mitosis. Thus, in late G2, the sudden increase in Cdk1 activity that coincides with entry into mitosis is thought to be achieved by a double positive feedback loop in which Cdk1 kinase activity both inhibits Wee1 kinase and activates Cdc25 phosphatase (9). The resulting flood of Cdk1 activity promotes mitosis by phosphorylating a range of nuclear substrates (e.g. lamin and histone H3) until its mitotic partner, cyclin B, is degraded at anaphase. The intrinsic cellular signals that determine when this switch is turned on during a normal cell cycle are unclear.

The in vivo importance of inhibitory phosphorylation of Cdk1 has been demonstrated by experiments involving mutant Cdk1 proteins lacking phosphorylation sites. Fission yeasts with a Y15F mutation advance prematurely into mitosis (10), whereas vertebrate cells overexpressing Cdk1 with T14A and Y15F substitutions (Cdk1-AF) undergo mitotic catastrophe (i.e. entry into mitosis before DNA replication is complete, resulting in aberrant mitosis and cell death) (11, 12). In a variety of systems, mutation of Cdk1 inhibitory phosphorylation sites also impairs G2 arrest in response to genotoxic damage, indicative of signaling pathways that sense such damage and culminate in the inhibitory phosphorylation of Cdk1 (13–15). Despite the crucial role that inhibitory phosphorylation of Cdk1 undoubtedly plays in regulating the G2/M transition, it remains unclear whether the autocatalytic burst of Cdk1 activity that accompanies Cdk1 dephosphorylation is absolutely required to promote the G2/M transition or whether relief from other inhibitory mechanisms can suffice.

Another conserved role for Cdk1 is to ensure that S phase is dependent on completion of a preceding mitosis. This role was first revealed in fission yeast after inactivation of Cdk1/cyclin B; cells failing to enter mitosis do not simply arrest in G2 but reset in G1 and enter successive but discrete rounds of S phase, leading to cells with ploidies of 4, 8, 16 N, etc. (16–18). A similar
endoreduplication phenotype was observed when Cdk1 was depleted in human cells by genetic means (19). In fission yeast, Cdk1 binds at replication origins and prevents the formation of prerelocplexes, apparently by phosphorylating key components of the prereplication complex (20), and various components of the mammalian prereplication complex are also known to be phosphorylated by Cdk1 (21, 22). Thus, assuming that the serine kinase activity of Cdk1 is required to suppress endoreduplication during S and G₂, the question arises of how this is achieved without promoting premature mitosis.

To address questions of this kind, it is useful to have a genetic system in which the functionality of variant Cdk1 proteins can be analyzed in cells depleted for endogenous Cdk1. The human HT2-19 cell line is uniquely suited to this purpose (19). HT2-19 cells have one inactivated CDK1 allele and one allele that is regulated by the lac repressor. In the presence of IPTG, Cdk1 protein levels and kinase activity in HT2-19 cells are 30–40% of those in parental HT1080 cells but sufficient for cell proliferation, albeit with an increased doubling time. After removal of IPTG, Cdk1 protein levels and kinase activity decline to <10% of +IPTG values, and appreciable endoreduplication is observed, accompanied by apoptosis. Because they can be rescued from IPTG dependence by transfection with a Cdk1 expression plasmid (19), HT2-19 cells can be used in complementation tests to assess the functionality of variant Cdk1 proteins. In the present study, we find that the Cdk1 from Schizosaccharomyces pombe (Cdc2) cannot rescue HT2-19 cells from IPTG dependence, whereas human Cdk1-AF can. Further analysis reveals that the latter complementation requires a small amount of residual endogenous Cdk1 expression, without which cells fail to divide or endoreduplicate. These results indicate that some, but not all, cellular Cdk1 must be susceptible to inhibitory phosphorylation for a successful G₂ to M transition. The results also suggest a model in which two thresholds of Cdk1 activity must be exceeded sequentially during the cell cycle, the first to inhibit endoreduplication and the second to promote entry into mitosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Electroporation**—HT1080 and HT2-19 cell culture and stable transfection by electroporation using a Gene Pulser electroporator (Bio-Rad) were as described (19). Where required, doxycycline was added to media at 1 μg/ml. To select for colonies stably transfected with pZeoSV derivatives, 200 μg/ml zeocin was added to the medium 48 h after transfection. Where required, IPTG was added to the medium to a final concentration of 0.2 mM.

**Plasmids**—pBSgpt and pBSgptCDC have been described previously (19). The sequence immediately upstream of the CDK1 start codon in pBSgptCDC was mutated (from CTAATG to CATATG) to create an Ndel site. pBSgptCDK1 WT-HA and pBSgptCDK1 AF-HA were made as follows. pBSgptCDC was digested with Ndel and end-filled; the ~1-kb Cdk1 coding fragment was discarded, and the remaining 10.5-kb fragment was ligated to the 1-kb BamHI fragment of pUHD10-3-CDC2-HA or pUHD10-3-CDC2-A14F15-HA (kindly donated by David Morgan, University of California, San Francisco) carrying the Cdk1 coding sequence modified as described (23). To make pBSgptcdc-sp, a 1-kb BamHI fragment from pTZ19R.cdc2 (kindly donated by Chris Norbury, Oxford University, UK) and the 10.5-kb Ndel fragment of pBSgptCDC were end-filled and ligated. To make pTetCDK1 WT-HA–zeo and pTetCDK1 AF-HA–zeo, a 1.3-kb EcoRV/PvuII fragment from pCMVzeo (Invitrogen) was ligated to Spsl-linearized pUHD10-3CDC2-HA or pUHD10-3-CDC2-A14F15-HA, respectively. For all plasmids, the correct orientation and sequence of CDK1 inserts were confirmed by restriction enzyme digests and DNA sequencing, respectively. A CDK1 gene-targeting construct, pCDC/zeo, was made by inserting an end-filled 1.3-kb BamHI–Spel fragment from pZeoSV (Invitrogen) into pCDC2AX (19), linearized at an Asp718 site at the beginning of CDK1 exon 3.

**Kinase Assays**—Cdk1 kinase assays were essentially as described previously (19), using either a rabbit antibody (kindly donated by Chris Norbury, Oxford University) to the human Cdk1 C-terminal peptide LDNQIKKM or a rat anti-hemagglutinin antibody (13CA5; Roche Applied Sciences). Each immunoprecipitation (100 μl) contained 100 μg of cell lysate protein.

**Immunoblots**—Immunoblots were as described previously (19). For Cdk1, a primary monoclonal antibody (SC-54; 1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a secondary, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody (P0447; 1:1000 dilution; Dako) were used. For actin, a primary rabbit antibody (A-2066; 1:1000 dilution; Sigma) and secondary horseradish peroxidase-conjugated rat anti-rabbit immunoglobulin antibody (P0448; Dako) were used.

**Flow Cytometry**—Flow cytometric analysis of nuclei for DNA content was as described previously (19).

**Gene Targeting**—The CDK1 gene targeting construct pCDC/zeo (10 μg) was linearized at a unique Asp718 site and electroporated into clone AF18 cells. Zeocin-resistant colonies were screened by a PCR assay for targeted integration at the Cdk1 allele using primers 5’-CCTTTGTCAATCAATCAAGAG-3’ (CDK1 gene) and 5’-ATACGTAGTAGTACTGCCA-3’ (zeocin cassette). PCR was performed on pellets of 1000–10,000 cells as described previously (19). To determine whether the desired (functional) CDK1 allele had been targeted, thereby removing all endogenous Cdk1 expression, PCR-positive clones were analyzed by immunoblotting with Cdk1 antibody.

**RNA Interference**—RNA oligonucleotide pairs specific for endogenous Cdk1 (5’-GAUGUAGGUUCUUCAGAAACAAA-3’ and 5’-UUUGUCAGAAAGCUAUCUUC-3’) or GFP (5’-GCUAUCGUCCAGGAGCCAC-3’ and 5’-UGUCCUCCAUGACGCUUUCUU-3’) were obtained preannealed from Dharmacon and delivered to cells by Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were plated at 50% confluence in 2- or 3.5-cm diameter wells in antibiotic-free medium the day before transfection. Unless indicated otherwise, HT2-19 cells were maintained in medium with IPTG. HT2-19 derivatives were maintained in medium without IPTG. The following day, a mixture containing 600 nM siRNA and Oligofectamine (3% [v/v]) in Opti-MEM was prepared according to the manufacturer’s instructions and added to cells (100 or 500 μl/well) with 5 volumes of antibiotic-free medium, to give a final siRNA concentration of 100 nM. Fixing,
staining and microscopy of cells was as previously described (19).

RESULTS

Human Cdk1-AF Rescues HT2-19 Cells from Cdk1 Depletion

A plasmid (pBSgptCDC) carrying the human Cdk1 cDNA driven by the CDK1 promoter and the bacterial gpt gene, which confers resistance to mycophenolic acid and xanthine (MPA/X), was previously shown to rescue HT2-19 cells from IPTG dependence (19). Derivatives of pBSgptCDC were made to encode human Cdk1 with a C-terminal hemagglutinin (HA) tag and either with (pBSgptCDK1-AF-HA) or without (pBSgptCDK1-WT-HA) accompanying T14A and Y15F mutations. A further pBSgptCDC derivative (pBSgptcdc-sp) was made to express the only Cdk of S. pombe, Cdc2. Four complementation experiments were carried out (Table 1) to test one or more of these plasmids or empty vector (pBSgpt) expressing only the gpt gene for their ability to rescue HT2-19 cells from IPTG dependence. Electroporated HT2-19 cells were selected for gpt expression (in MPA/X and IPTG) or for both gpt expression and IPTG independence (in MPA/X alone). Spontaneous IPTG independence in HT2-19 (19) (as indicated by IPTG-independent colonies appearing after transfection with pBSgpt) occurred to varying extents but was sufficiently infrequent to allow the detection of IPTG independence conferred by exogenous Cdk1 expression. Thus, above background levels of IPTG independence were clearly conferred by pBSgptCDK1-WT-HA and, unexpectedly, by pBSgptCDK1-AF-HA. When expressed as a percentage of all gpt+ colonies, the frequencies of IPTG independence conferred by pBSgptCDK1-WT-HA and pBSgptCDK1-AF-HA were similar (14 versus 24% and 23 versus 17% in experiments 1 and 3, respectively). Within the sensitivity of the complementation assay, Cdk1-AF-HA and Cdk1-WT-HA therefore appear to compensate for Cdk1 depletion with very similar efficiencies. The expression plasmid for Cdc2 (pBSgptcdc-sp) was unable to confer above background levels of IPTG independence (Table 1, experiment 2). This was confirmed when none (0 of 45) of the MPA/X resistance colonies of experiment 4 (Table 1) survived in the absence of IPTG, although most (3 of 4) of the colonies tested by immunoblotting were shown to express the S. pombe protein (not shown).

Cdk1-WT-HA and Cdk1-AF-HA Expression in IPTG-independent HT2-19 Transfectants—To confirm that exogenous Cdk1 was expressed in IPTG-independent transfectants and that endogenous Cdk1 expression was still IPTG-dependent, several IPTG-independent clones from experiment 2 (Table 1) were analyzed by immunoblotting with a Cdk1 antibody (Fig. 1). As expected from previous studies (12, 15, 24), both endogenous and exogenous Cdk1 were detectable as a triplet of bands, the latter triplet migrating more slowly than the former because of the HA tag and, for clones transfected with CDK1-AF expression plasmid, lacking the upper two bands that represent Thr14- and Tyr15-phosphorylated protein. Thus, six bands (two triplets) were detected in pBSgptCDK1-WT-HA-rescued

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TABLE 1

| Complementation tests in HT2-19 |
|-------------------------------|
| Plasmid | n | Colonies surviving selection | Selection ratio | Transfection efficiency |
|---------|---|-----------------------------|-----------------|-------------------------|
|         |   | MPA/X/IPTG | MPA/X | % | × 10^-4 |
|---------|---|------------|-------|---|--------|
| Experiment 1 | | | | | |
| pBSgpt  | 10 | 42 | 2 | 5 | 0.08 |
| pBSgptCDK1-WT-HA | 10 | 78 | 19 | 24 | 0.16 |
| pBSgptCDK1-AF-HA | 10 | 83 | 12 | 14 | 0.17 |
| Experiment 2 | | | | | |
| pBSgpt  | 6 | NA | 2 | NA | NA |
| pBSgptCDK1-WT-HA | 6 | NA | 35 | NA | NA |
| pBSgptCDK1-AF-HA | 6 | NA | 28 | NA | NA |
| pBSgptcdc-sp | 6 | 78 | 3 | 3.8 | 2.5 |
| Experiment 3 | | | | | |
| pBSgpt  | 3.5 | 119 | 0 | 0 | 0.68 |
| pBSgptCDK1-WT-HA | 3.5 | 76 | 13 | 17 | 0.43 |
| pBSgptCDK1-AF-HA | 3.5 | 30 | 7 | 23 | 0.17 |
| Experiment 4 | | | | | |
| pBSgptcdc-sp | 6 | >300 | NA | NA | >0.5 |

*a Number of HT2-19 cells electroporated (×10^-6).
*b Number of colonies generated in the indicated selection conditions (half of the electroporated cells were used for each selection).
*c Number of colonies in column 4 as a percentage of those in column 3.
*d Number of colonies in column 3 divided by number of cells plated.
e NA, not available.
clones (clones WT1, WT2, etc.), whereas only the four fastest migrating of these were detected in pBSgptCDK1-AF-HA-rescued clones (clones AF8, AF14 etc.) In most clones, endogenous Cdk1 bands were also distinguishable from exogenous Cdk1 bands by their dependence on IPTG. A few clones (e.g. WT1 and AF22) had, however, lost IPTG-dependent expression of endogenous Cdk1, suggestive of a spontaneous rever- sion event, as previously noted (19). Interestingly, although some of the Cdk1-WT-HA-rescued clones (e.g. WT7 and WT9) expressed substantially more exogenous than endogenous Cdk1, this was not the case for any of the Cdk1-AF-HA-rescued clones. Indeed, the amount of Cdk1-AF-HA protein relative to endogenous Cdk1 was remarkably similar between clones, consistent with the idea that excessive expression of Cdk1-AF-HA is not tolerated by cells, whereas a minimum level is required to compensate for endogenous Cdk1 depletion. These results supported the conclusion from the complementation analysis (Table 1) that Cdk1-AF-HA, as well as Cdk1-WT-HA, is capable of compensating for the Cdk1 deficiency in HT2-19 cells grown in the absence of IPTG.

Histone H1 Kinase Activity in IPTG-independent HT2-19 Transfectants—To confirm that exogenous Cdk1 was active in the rescued clones, histone H1 kinase assays were carried out on immunoprecipitates of two Cdk1-WT-HA-rescued clones (WT2 and WT3) and two Cdk1-AF-HA-rescued clones (AF18 and AF29) that had been treated with or without nocodazole (Fig. 2). By inhibiting microtubule polymerization, an 8-h treatment with nocodazole increases the mitotic index of cultures ∼5-fold (data not shown). Similar amounts of anti-HA-precipitable H1 kinase activity were detected in all four clones, but the H1-kinase activity was substantially increased by nocodazole treatment of clones WT2 and WT3, whereas no such increase was seen for clones AF18 and AF29 (Fig. 2A). Similar results were obtained when separate lysates were prepared and immunoprecipitated with a different anti-HA antibody (supplementary Fig. 1S). These results confirm that Cdk1-WT-HA, like endogenous Cdk1, is activated by loss of inhibitory phosphorylation upon entry into mitosis, whereas Cdk1-AF-HA is not. Endogenous H1 kinase activity, as detected in anti-Cdk1 immunoprecipitates, was similar in all four rescued clones, being low but detectable and noticeably elevated in samples from nocodazole-treated cells (Fig. 2B). The Cdk1 antibody does not immunoprecipitate HA-tagged Cdk1, because it recognizes a C-terminal epitope that is disrupted by the addition of the HA tag. The fact that the HA-associated activity before nocodazole treatment is similar in all four rescued clones is consistent with the similar amounts of non-phosphorylated exogenous protein, relative to endogenous Cdk1 protein, seen in these clones (Fig. 1).

Cdk1-AF-HA Expression Is Tolerated by HT1080 Cells but Is Selectively Disadvantageous—The ability of Cdk1-AF-HA to complement the Cdk1 deficiency in HT2-19 cells was a surprise, given the published data describing the toxic effects of transient Cdk1-AF expression. To determine whether the apparent tolerance of Cdk1-AF-HA expression was a pecu- liarity of the host cells, parental HT1080 cells expressing the doxycycline-regulated transcriptional activator tTA (HTET) (25) were transfected with plasmids (pTetCDK1-WT-HA-zeo and pTetCDK1-AF-HA-zeo) carrying an expression cassette for zeocin resistance (zeo') and tTA-responsive expression cassette for Cdk1-WT-HA or Cdk1-AF-HA (Fig. 3A). After transfection of pTetCDK1-WT-HA-zeo, induction of Cdk1-WT-HA expression by removal of Dox increased the frequency of zeocin-resistant colonies recovered (Table 2). This result can be explained if induction of the tTA binding to the promoter/enhancer driving Cdk1-WT-HA expression also enhances expression of the zeo' cassette. After transfection of

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**FIGURE 2.** Histone H1 phosphorylation assays in immunoprecipitates from HT1080 and IPTG-independent HT2-19 derivatives. Lysates were prepared from cells that had been grown continuously without IPTG and treated with nocodazole for the indicated number of hours. Equal amounts of cell lysate (100 μg of protein) were immunoprecipitated with antibody to hemagglutinin (A) or Cdk1 (B), incubated with [γ-32P]ATP and histone H1. 32P-Labeled histone H1 was visualized by SDS-PAGE and autoradiography.

**FIGURE 3.** Inducible expression of Cdk1-WT-HA or Cdk1-AF-HA in HT1080 cells. A, structure of plasmids (pTetCDK1-WT-zeo and pTetCDK1-AF-zeo) used for inducible expression of HA-tagged Cdk1. White boxes within gray arrows represent open reading frames within transcribed sequences. For human CDK1, residues 14 and 15 are indicated (TY or AF). The thin black lines represent other vector sequences with positions of SV40 t-antigen splice and polyadenylation sites indicated (pA). PvuI sites (P) were used for linearization. B, immunoblot analysis with a Cdk1 antibody of histone H1 kinase activity in immunoprecipitates, was similar in all four rescued clones, consistent with the similar amounts of non-phosphorylated exogenous protein, relative to endogenous Cdk1 protein, seen in these clones (Fig. 1).
pTetCDK1-AF-HA-zeo, however, induction of Cdk1-AF-HA expression decreased the frequency of zeo\(^r\) colonies recovered (Table 2). Furthermore, immunoblotting of zeo\(^r\) colonies selected and expanded in the absence of doxycycline (i.e. while CDK1-WT-HA or CDK1-AF-HA cassettes were expressed) showed the former to express detectable, although variable, levels of Cdk1-WT-HA, whereas the latter expressed no detectable Cdk1-AF-HA (Fig. 3). Expression of Cdk1-AF-HA could nevertheless be detected in immunoblots of zeo\(^r\) clones selected and expanded in the presence of doxycycline (i.e. to suppress expression from CDK1-WT-HA or CDK1-AF-HA cassettes) and then grown without doxycycline for 5 days (Fig. 3C), during which time cells appeared normal and continued to proliferate. Together, these observations suggest that HT1080 cells expressing physiological levels of Cdk1-AF-HA are able to proliferate effectively but that they are at a selective disadvantage and gradually outgrown by derivatives in which Cdk1-AF-HA expression is suppressed. The persistent expression of Cdk-AF-HA in HT2-19 derivatives selected in the absence of IPTG is therefore evidence that, under these conditions, Cdk-AF-HA confers a selective advantage.

**Growth of IPTG-independent HT2-19 Clones**—Four rescued clones (WT2, WT3, AF18, and AF29) were selected for further analysis. All but WT2 reproducibly grew faster and formed colonies more efficiently when IPTG was restored to the medium (Fig. 4, A and B). This implied that for such clones there is a selective advantage in the absence of IPTG to any cells that up-regulate their endogenous Cdk1 expression, something we knew HT2-19 cells were capable of doing at low frequencies (19). This may explain why endogenous Cdk1 expression in some clones had already lost IPTG dependence at the time of first analysis (e.g. clones WT1 and AF22; Fig. 1). It may also explain why we found that clones such as AF18, whose endogenous Cdk1 was still IPTG-dependent when first isolated (Fig. 1), had lost this dependence in later passages (Fig. 4C). The fact that expression of exogenous Cdk1-AF-HA is maintained in clones such as AF18, despite evidence that its expression in HT1080 cells is selectively disadvantageous (Fig. 3), suggests that exogenous Cdk1 activity remains advantageous to HT2-19 cells even when endogenous Cdk1 is expressed. This is consistent with the fact that, even in the presence of IPTG, the cell doubling time and amount of Cdk1 protein are both reduced in HT2-19 compared with parental HT1080 cells (19).

**Cdk1-AF-HA-rescued Clones Depend on Residual Endogenous Cdk1**—Based on the considerations above, it was apparent that residual endogenous Cdk1 expression in the absence of IPTG, even when insufficient to support HT2-19 viability, might cooperate with exogenous Cdk1 to rescue HT2-19 cells. We therefore wanted to know whether residual endogenous Cdk1 expression was required for Cdk1-AF-HA (but not Cdk1-WT-HA) to rescue HT2-19 cells from IPTG dependence. To test this, we attempted to disrupt the IPTG-regulated CDK1 allele in clone AF18 by use of a gene targeting construct (pCDCzeo) analogous to that used previously to disrupt the other allele (19) but with a different selectable marker. Approximately 1000 drug-resistant transfectants were screened by PCR for disruption, but of the three PCR-positive clones identified, none had targeted the IPTG-regulated allele (see “Experimental Procedures”). We therefore used a short interfering RNA (siRNA\(^{CDK1}\)) designed to silence expression of endogenous CDK1 without affecting expression of the genes encoding HA-tagged Cdk1 (Fig. 5A). Immunoblot analyses of HT2-19, WT4, and AF18 cells transfected with either siRNA\(^{CDK1}\) or a control siRNA confirmed that endogenous but not exogenous Cdk1 was depleted by siRNA\(^{CDK1}\) (Fig. 5B), despite the appearance, for unknown reasons, of a nonspecific band (stars). The effects of siRNA\(^{CDK1}\) on cell appearance, proliferation, and ploidy were assessed and found to be different for each cell line (Fig. 6). HT2-19 cells grown in IPTG and treated with siRNA\(^{CDK1}\) showed cell enlargement, reduced proliferation, and increased ploidy compared with cells treated with control siRNA (Fig. 6). HT2-19 cells therefore respond to siRNA\(^{CDK1}\) much as they do to removal of IPTG. In contrast, the appearance, proliferation, and ploidy of WT4 cells were all relatively unaffected by siRNA\(^{CDK1}\), whereas in AF18 cells proliferation was impaired, but with no signs of cell enlargement or increasing ploidy. These results suggest that, in the absence of residual endogenous Cdk1, Cdk1-AF-HA is unable to promote mitosis but able to suppress endoreduplication, whereas Cdk1-WT-HA is capable of both functions.

Endoreduplication was also assessed 5 days after siRNA transfection by fluorescence microscopy of 4′,6-diamidino-2-phenylindole, hydrochloride-stained nuclei (Fig. 7). Greatly enlarged and distorted nuclei, typical of endoreduplicating cells and similar to those that develop after IPTG withdrawal from HT2-19, were clearly seen in siRNA\(^{CDK1}\)-transfected but not siRNA\(^{GFP}\)-transfected HT2-19 cells. Under the same conditions, however, similar evidence of endoreduplication was not seen in any of the HT2-19 derivatives, WT2, WT4, AF18, and AF19. Furthermore, it was again apparent that cell proliferation was impaired by siRNA\(^{CDK1}\) in Cdk1-AF-HA-expressing clones (AF18 and AF29) but not in Cdk1-WT-HA-expressing clones (WT2 or WT4).

**DISCUSSION**

The data presented here show that Cdk1 that lacks inhibitory phosphorylation sites is able to rescue a human cell line ren-

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### Table 2

Transfection to zeocin resistance with or without CDK1 expression

| Experiment | Plasmid | n^a | Zeo\(^r\) colonies | Ratio |
|------------|---------|-----|---------------------|-------|
|            |         |     | With doxycycline    | Without doxycycline |
| Experiment 1 | pTetCDK1-WT-HA-zeo | 15 | 877 | 1096 | 1.25 |
|            | pTetCDK1-AF-HA-zeo | 15 | 501 | 341 | 0.68 |
| Experiment 2 | pTetCDK1-WT-HA-zeo | 7.5 | 338 | 349 | 1.03 |
|            | pTetCDK1-AF-HA-zeo | 7.5 | 92 | 39 | 0.42 |
| Experiment 3 | pTetCDK1-WT-HA-zeo | 7.5 | 129 | 143 | 1.11 |
|            | pTetCDK1-AF-HA-zeo | 7.5 | 21 | 5 | 0.24 |
| Av         | pTetCDK1-WT-HA-zeo | | | 1.13 |
|            | pTetCDK1-AF-HA-zeo | | | 0.45 |

^a Number of HTET cells electroporated (×10^\(^5\)).
dered nonviable by depletion of its endogenous Cdk1. On its own, this is a surprising result for at least two reasons. First, the sudden release from inhibitory phosphorylation is a characteristic and highly conserved feature of the G2/M transition generally regarded as being essential for the normal G2/M transition. Second, previous experiments have shown that loss of the inhibitory phosphorylation of Cdk1 leads to mitotic catastrophe and should not therefore be compatible with cell viability (11, 12). As already noted, however, other mechanisms exist for limiting Cdk1 activity, such as Cdk inhibitors and cytoplasmic sequestration, and these could in principle be used to prevent premature mitosis by Cdk1 that is not subject to inhibitory phosphorylation. Furthermore, the levels of Cdk1-AF expression used to generate mitotic catastrophe were unnaturally high, allowing for the possibility that more physiological levels can be tolerated. More modest levels of Cdk1-AF were relatively well tolerated in HeLa cells, at least in the short term (13, 15).

On the basis of the above arguments, the case could be made that inhibitory phosphorylation serves a redundant function during the normal mammalian cell cycle, as has been described for budding yeast (26). We could not discount an essential role for inhibitory phosphorylation, however, because residual endogenous Cdk1 kinase activity that was still subject to inhibitory phosphorylation was detectable in Cdk1-AF-rescued cells (Fig. 2). We considered the possibility that such residual activity was necessary, in combination with the constitutive Cdk1-AF kinase activity, for survival of clones, such as AF18. To test this,
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model proposed by Stern and Nurse (27) to explain how a single Cdk1/cyclin in yeast could alternately promote both S phase and M phase in yeast. In that model, rising Cdk1 activity, following cyclin degradation in late mitosis, promotes S and M phases when lower and upper activity thresholds are crossed, respectively. As already noted, in higher eukaryotes, the role of Cdk1 in promoting S phase has been adopted by other Cdk proteins, whereas its role in preventing endoreduplication appears to have been conserved. The mechanism by which Cdk1 prevents endoreduplication is uncertain but is distinct from the mechanism that prevents multiple replication origin firing within a single S phase, because endoreduplicating Cdk1-impaired cells still display well defined S phases (17, 19, 28). In fission yeast, the mechanism requires Cdk1 binding to the origin of replication complex, where it may prevent the licensing of replication origins by phosphorylating origin of replication complex proteins, such as Orp2 (20). By assuming that a similar mechanism exists in mammalian cells and suggesting that a threshold of Cdk1 activity is required, we cannot only explain the results we have observed (Fig. 8) but also provide a possible answer to the question of how Cdk1 kinase activity can suppress endoreduplication without promoting mitosis. Although it is consistent with much of the data, this two-threshold model remains speculative, and further work, beyond the scope of this paper, is required to test its validity in more detail.

A recent publication (29) described the formation of Cdk2-cyclin B complexes upon depletion of Cdk1 by siRNA in HeLa cells, suggesting that there may be some functional redundancy between Cdk1 and Cdk2. It is possible that Cdk2/cyclin B forms in HT2-19 cells in response to the disruption of one Cdk1 allele or to repression of the other. Any such activity is, however, clearly insufficient to compensate for the depletion of Cdk1 in HT2-19 cells when IPTG is removed. Furthermore, Cdk2/cyclin B is unlikely to play a role in clones rescued by expression of Cdk1-WT-HA or Cdk1-AF-HA, because the exogenous Cdk1 will presumably form complexes with Cyclin B at the expense of any Cdk2/cyclin B.

Human Cdk1 was originally identified by its ability to complement a temperature-sensitive cdc2 mutation in fission yeast (30), demonstrating a remarkable degree of functional conservation between the yeast and human proteins. Here we were able to carry out the converse experiment and found that cdc2 was unable to complement HT2-19 cells grown without IPTG. Further work is required to determine whether this represents an inability of the yeast protein to associate with essential acti-
mators (e.g. cyclin B) in human cells or whether it can form active complexes that are inappropriately regulated, causing, for example, premature entry into S phase. The latter possibility is raised not only by the fact that the yeast protein participates in the G1 to S transition in its normal host but also because, in the absence of Cdk2, mammalian Cdk1 can associate with cyclin E and promote the G1 to S transition (31).

The analyses of Cdk1 inhibitory phosphorylation described here were part of a more general analysis of the suitability of HT2-19 cells as hosts for the in vivo analysis of variant Cdk1 function. Previous in vivo analyses of higher eukaryote Cdk function have involved expression in yeast conditional Cdk mutants (32, 33) or in mammalian cells that continue to express endogenous Cdk1, both of which approaches have obvious limitations (11, 12, 15, 34). HT2-19 cells allow variant Cdk1 function to be assessed in cells depleted of endogenous Cdk1. Thus, we were able to test for the first time whether yeast Cdk1 (Cdc2) or human Cdk1-AF could compensate for missing Cdk1. The results showed the HT2-19 complementation system to be effective and informative. In the case of Cdk1-AF analyses, however, the results were complicated by the existence of residual Cdk1 activity in the absence of IPTG. Although these complications proved to be interesting in themselves, future studies of mutant Cdk1 function would benefit from a cell line in which Cdk1 can be more completely depleted. This can be achieved by...
the use of a more stringent regulatory system than the LacR used in HT2-19 cells, such as tetracycline-regulated transcription (35), as recently used for the analysis of mutant topoisomerase II function in HT1080 cells (25).

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