Chk1 fusion protein or unfused GST were analyzed by glutathione (GSH)-Sepharose precipitation followed by immunoblotting. GST-Chk1 precipitated with Cdc25, whereas no Cdc25 was detected in association with GST (Fig. 3A). Incubation of GST-Chk1 with associated Cdc25 in the presence of [γ-^{32}P]adenosine triphosphate (ATP) resulted in phosphorylation of Cdc25 (Fig. 3B), suggesting that Cdc25 may be a direct substrate of Chk1 kinase. Chk1 protein purified from an insect cell expression system phosphorylated Cdc25 in vitro (*21*).

Activation of the DNA damage checkpoint requires Rad3, a kinase related to the ATM protein that is defective in ataxia telangiectasia patients (*3*). DNA damage leads to increased phosphorylation of Chk1 by a Rad3-dependent process, suggesting that Chk1 may be activated by phosphorylation (*5*). Our studies identify Cdc25 as a key, possibly direct, target of Chk1. In addition, these findings exclude Wee1 as an important Chk1 substrate. Therefore, we propose that Rad3-dependent activation of Chk1 leads to negative regulation of Cdc25 (Fig. 4). This negative regulation may occur by direct inhibition of Cdc25 activity, prevention of the activation of Cdc25 that occurs at the G_2-M transition, or interference in the interaction between Cdc25 and Cdc2. Inhibitory phosphorylation of Cdc2 is crucial for G_2 DNA damage arrest in mammalian cells (*22, 23*). In these cells it is not known whether this arrest is brought about by inhibition of Cdc2 dephosphorylation, nor is it known if mammals have a Chk1 homolog. However, in view of the striking degree of homology of mitotic control mechanisms in fission yeast and mammals, we expect that the *S. pombe* checkpoint control will serve as a useful paradigm for investigating the DNA damage checkpoint mechanism in more complex organisms.

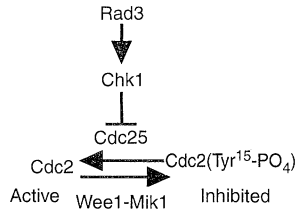

Fig. 4. Model of the DNA damage checkpoint mechanism in fission yeast. Rad3 and Chk1 kinases are required for the checkpoint. Chk1 undergoes a Rad3-dependent phosphorylation in irradiated cells, and Chk1 overexpression induces cell cycle arrest by a Rad3-independent mechanism (*20*), indicating that Chk1 activation is regulated by Rad3, perhaps by direct phosphorylation. Chk1 inhibits Cdc25 and thereby prevents Cdc2 Tyr^{15} dephosphorylation.

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24. *Schizosaccharomyces pombe* strains of the following genotypes were used: PR754, *wee1-50 mik1::ura4*$^+$; NR1604, *wee1-50 mik1::ura4*$^+$ *chk1::ura4*$^+$; PR109, wild-type; GL192, *cdc2-3w cdc25::ura4*$^+$; BF1921, *nmt1:GST-chk1:leu1*$^+$; BF1910, *cdc2-3w cdc25::ura4*$^+$ *nmt1:GST-chk1:leu1*$^+$; BF1911, *cdc2-3w nmt1:GST-chk1:leu1*$^+$; NR1970, *wee1::ura4*$^+$ *nmt1:GST-chk1:leu1*$^+$; BF1920, *nmt1:GST:leu1*$^+$ *cdc25:6HA*; and BF1915, *nmt1:GST-chk1:leu1*$^+$ *cdc25:6HA*. All strains were *leu1-32 ura4-D18*. All *nmt1* promoter constructs were integrated at the *leu1* locus. Growth media and general methods for *S. pombe* have been described [S. Moreno, A. Klar, P. Nurse, *Methods Enzymol.* **194**, 795 (1991)]. Cells were synchronized by centrifugal elutriation with a Beckman JE-5.0 elutriation rotor. Five minutes after elutriation, half of the cells were irradiated with gamma radiation from a ^{137}C source at 3 Gy min^{-1} at room temperature, which ranged from 23° to 25°C. After 35 min of irradiation at room temperature, half of each of the irradiated and unirradiated cultures were shifted to 35°C. To continue radiation exposure at 35°C, we alternatively irradiated the 35°C irradiated culture for 20 min and then incubated it at 35°C for 20 min. The temperature of this culture varied between 32° and 35°C. The number of cells having passed mitosis was determined by microscopic observation of the number of cells that had begun or finished septation, divided by the total number of cells.
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27. Purification of GST proteins expressed in fission yeast, immunoblotting, protein kinase assays, and immunoprecipitation were performed as described [K. Shiozaki and P. Russell, *Nature* **378**, 739 (1995); *Genes Dev.* **10**, 2276 (1996); S. Moreno, P. Nurse, P. Russell, *Nature* **344**, 549 (1990)]. Control experiments with *cdc2-3w* Δ*cdc25* cells that expressed GST-Chk1 confirmed that Cdc25 was specifically associated with GST-Chk1 and phosphorylated in association with GST-Chk1. Analysis of the GST-Chk1 kinase reaction mixture before immunoprecipitation with antibody to Cdc25 revealed proteins migrating with the predicted size of GST-Chk1 and Cdc25 as the major ^{32}P-labeled protein.

28. We thank O. Mondesert for technical assistance; C. McGowan and K. Shiozaki for technical advice; S. Forsburg and A. Carr for strains; S. Reed and I. Wilson for antibody reagents; and members of the Scripps Cell Cycle Groups. N.R. was supported by an NIH postdoctoral fellowship. This work was funded by NIH.

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Conservation of the Chk1 Checkpoint Pathway in Mammals: Linkage of DNA Damage to Cdk Regulation Through Cdc25

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In response to DNA damage, mammalian cells prevent cell cycle progression through the control of critical cell cycle regulators. A human gene was identified that encodes the protein Chk1, a homolog of the *Schizosaccharomyces pombe* Chk1 protein kinase, which is required for the DNA damage checkpoint. Human Chk1 protein was modified in response to DNA damage. In vitro Chk1 bound to and phosphorylated the dual-specificity protein phosphatases Cdc25A, Cdc25B, and Cdc25C, which control cell cycle transitions by dephosphorylating cyclin-dependent kinases. Chk1 phosphorylates Cdc25C on serine-216. As shown in an accompanying paper by Peng *et al.* in this issue, serine-216 phosphorylation creates a binding site for 14-3-3 protein and inhibits function of the phosphatase. These results suggest a model whereby in response to DNA damage, Chk1 phosphorylates and inhibits Cdc25C, thus preventing activation of the Cdc2–cyclin B complex and mitotic entry.

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In response to DNA

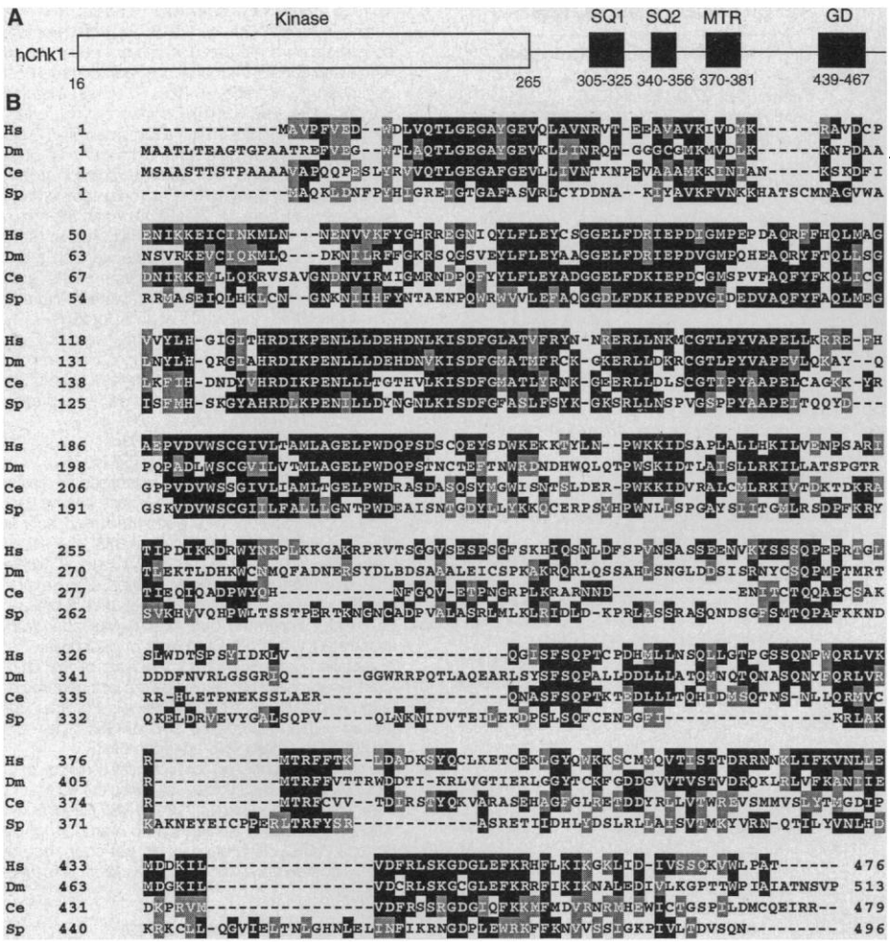

Fig. 1. Isolation of the human and mouse *CHK1* genes. (**A**) Domain structure of the predicted human Chk1 (hChk1) protein. The black boxes indicate regions of highest conservation. The GenBank accession number for hChk1 is AF016582, and for mChk1, AF016583. (**B**) Alignment of Chk1 homologs. Amino acid identities are shown as black boxes. Conservative changes are shown as shaded boxes. Hs is *Homo sapiens*, Sp is *S. pombe*, Ce is *C. elegans*, and Dm is *D. melanogaster*. The database DNA sequence for ceChk1 has a likely frame shift in the COOH-terminus. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

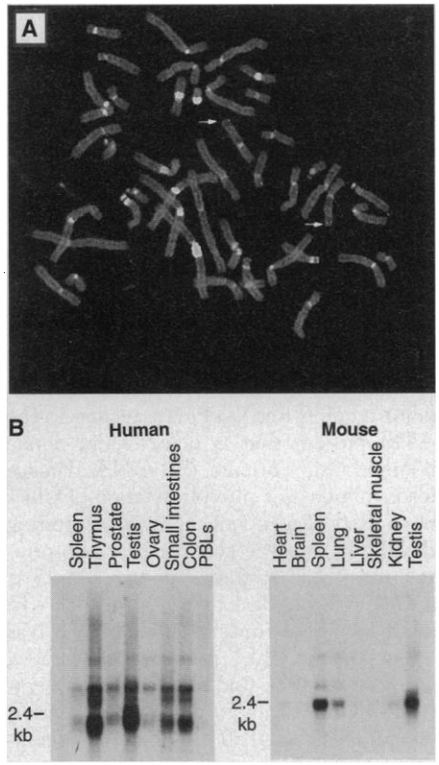

Fig. 2. Localization of *CHK1* on chromosome 11q24. (**A**) In situ hybridization was performed on mitotic chromosomes with fluorescently labeled human *CHK1* DNA. Arrows indicate *CHK1* localization at 11q24. (**B**) Northern analysis of human and mouse Chk1. Blots containing the polyadenylated RNA (2 μg per lane) from the indicated tissues were probed with human or mouse *CHK1* cDNAs.

damage, cells activate a checkpoint pathway that arrests the cell cycle to provide time for repair and induces the transcription of genes that facilitate repair. In yeast, this checkpoint pathway consists of several protein kinases including phosphoinositide (PI)–kinase homologs hATM, scMec1, and spRad3 and protein kinases scDun1, scRad53, and spChk1 (*1*) (the prefixes h, sc, and sp refer to *Homo sapiens*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*, respectively). In mammals, this pathway results in the activation of p53, which induces transcription of the cyclin-dependent kinase inhibitor $p21^{CIP1}$, resulting in arrest in the G_1 phase of the cell cycle (*2*).

To address the conservation of checkpoint function we searched for human homologs of yeast checkpoint genes. We used a degenerate polymerase chain reaction (PCR) strategy and identified a human gene very similar to the gene encoding Chk1 in *S. pombe* (Fig. 1) (*3*). With human *CHK1* cDNA as a probe, we isolated the gene encoding Chk1 from mouse (mChk1). The sequence of the longest human cDNA (1891 base pairs) predicted a translation product of 476 amino acids with a molecular size of 54 kD (Fig. 1A). No in-frame stop codon was found upstream of the first methionine, which is within the Kozak consensus sequence (*4*) and is likely to be the initiation codon because its encoded protein is the same size as that observed in cells (see below). The human *CHK1* gene is related to a *Caenorhabditis elegans* gene in the database and a *Drosophila melanogaster* gene *grp*, which has a role in cell cycle control and development (*5*) (Fig. 1B). The predicted hChk1 protein is 29% identical and 44% similar to spChk1, 40% identical and 56% similar to the ceChk1 (ce referes to *C. elegans*), and 44% identical and 56% similar to dmChk1 (dm refers to *D. melanogaster*). Sequence analysis revealed several COOH-terminal domains that are highly conserved in the Chk1 family of kinases (Fig. 1B).

The chromosomal location of *CHK1* was mapped to 11q24 by fluorescence in situ hybridization (Fig. 2A). This is adjacent to the gene encoding ATM at 11q23.

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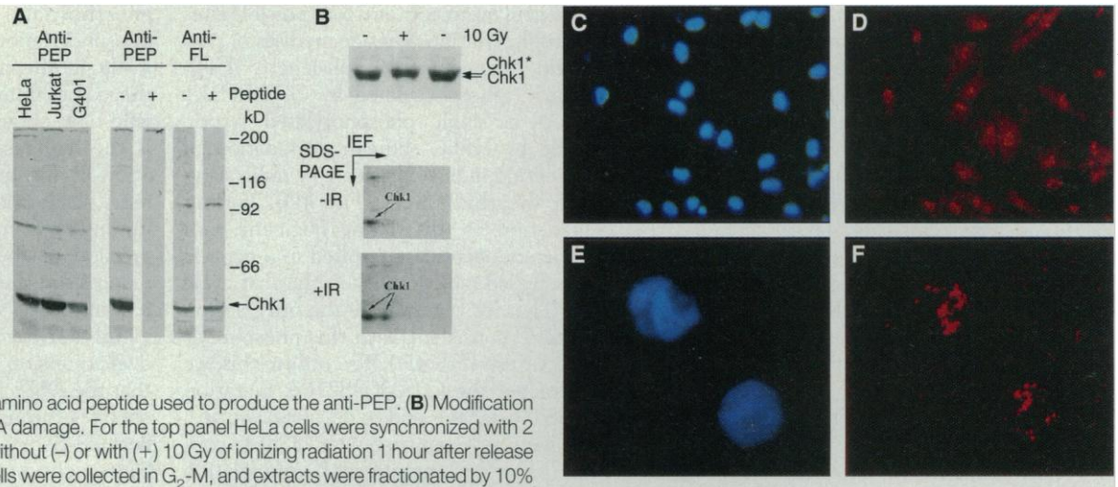

Fig. 3. Modification of the 54-kD nuclear hChk1 protein in response to DNA damage. (**A**) Protein from the indicated cell lines was fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with either affinity-purified antibodies to a COOH-terminal peptide (anti-PEP) or full-length hChk1 (anti-FL). HeLa extracts were immunoblotted with anti-PEP or anti-FL in the presence or absence of the 15–amino acid peptide used to produce the anti-PEP. (**B**) Modification of hChk1 in response to DNA damage. For the top panel HeLa cells were synchronized with 2 mM thymidine and treated without (–) or with (+) 10 Gy of ionizing radiation 1 hour after release from the thymidine block. Cells were collected in G_2-M, and extracts were fractionated by 10% SDS-PAGE and immunoblotted with anti-PEP. For the bottom panel Jurkat cells were treated (+IR) or not treated (–IR) with 10 Gy of ionizing radiation and incubated for 2 hours. Extracts from these cells were resolved in the first dimension by using isoelectric focusing (IEF) with pH 3 to 10 ampholytes, in the second dimension on a 10% SDS-PAGE, and immunoblotted with anti-PEP. (**C** to **F**) Punctate nuclear localization of hChk1. Human fibroblasts were fixed, stained with 4′,6′-diamidino-2-phenylindole (DAPI) to detect DNA, and probed with affinity-purified anti-PEP, biotinylated antibody to rabbit immunoglobulin G, and Texas Red streptavidin to reveal subcellular localization. (C) and (E), DAPI-stained nuclei; (E) and (F), hChk1 protein. Original magnifications: (C) and (D), ×40; (E) and (F), ×150.

Loss of heterozygosity at this region has been associated with a number of cancers including those of the breast, lung, and ovaries (6). Northern (RNA) blot analysis revealed ubiquitous expression of hChk1, with large amounts in human thymus, testis, small intestine, and colon (Fig. 2B). In adult mice, mChk1 was detected in all tissues examined and in large amounts in the testis, spleen, and lung (Fig. 2B). Mouse embryos from embryonic day 15.5 also revealed ubiquitous expression, with large amounts detected in the brain, liver, kidney, pancreas, intestines, thymus, and lung (7). Testis, spleen, and thymus also express large amounts of ATM (8).

Affinity-purified antibodies to hChk1 protein made in baculovirus (anti-FL) (9) or to its COOH-terminal 15 amino acids (anti-PEP) recognized a 54-kD protein (Fig. 3A) that comigrates with hChk1 expressed in baculovirus (7). The anti-PEP but not anti-FL signal was competed by addition of excess peptide, indicating that the two sera recognize different hChk1 epitopes, further confirming identity of the 54-kD band as endogenous hChk1. A 70-kD protein was also specifically recognized by anti-PEP. When mCHK1 was expressed from the cytomegalovirus (CMV) promoter in baby hamster kidney (BHK) cells, we detected a 54-kD nuclear protein only in transfected cells using antibodies to the COOH-terminal peptide of mChk1. This exogenous mChk1 comigrates with endogenous mChk1 from mouse lung tissue (7).

To determine whether hChk1 is modified in response to DNA damage like spChk1, we examined hChk1 protein in extracts from cells treated with ionizing radiation. hChk1 from extracts from damaged cells showed a minor but reproducible reduction in mobility compared with hChk1 from untreated cells (Fig. 3B). The change in mobility observed in response to DNA damage for spChk1 was also slight (*10*). This modification was confirmed by two-dimensional gel analysis, which demonstrated the generation of a more negatively charged hChk1 species 2 hours after gamma irradiation (Fig. 3B). These results indicate that hChk1 may participate in transduction of the DNA damage signal like spChk1. Indirect immunofluorescence revealed that hChk1 is localized to the nucleus in a punctate staining pattern (Fig. 3C), similar to that observed for ATM (8). mChk1 expressed in BHK cells confirmed the nuclear localization (7).

To test for the ability of hChk1 to regulate the cell cycle, we transfected hChk1 or hChk1(D130A), a catalytically inactive mutant, under the control of the CMV promoter or the CMV vector alone into HeLa cells treated with and without 6 Gy of ionizing radiation. We did not detect perturbation of the cell cycle by either kinase relative to vector alone, suggesting that overproduction alone was insufficient to deregulate the system (7).

Tyrosine phosphorylation of Cdc2 has been implicated in cell cycle arrest in response to DNA damage and replication blocks in both *S. pombe* (*11*, *12*) and humans (*13*). In *S. pombe*, Cdc2 mutants that cannot be phosphorylated on tyrosine are unable to arrest the cell cycle in response to blockade of DNA replication. Although it was originally thought that the DNA damage checkpoint did not operate through tyrosine phosphorylation, recent experiments have shown that tyrosine phosphorylation is required for *S. pombe* cells to arrest in response to DNA damage (*12*, *14*). Although it is now clear that tyrosine phosphorylation is required for proper checkpoint control, the experiments implicating tyrosine phosphorylation in this pathway do not distinguish between a regulatory role in which tyrosine phosphorylation rates are manipulated by the checkpoint pathways, or a passive role in which tyrosine phosphorylation is required to allow cell cycle arrest but is not the actual target of the checkpoint pathway (*1*, *15*).

To address this issue, we analyzed the ability of hChk1 to phosphorylate key regulators of Cdk tyrosine phosphorylation, the Cdc25 dual-specificity phosphatases hCdc25A, hCdc25B, and hCdc25C. These regulators were singled out for several reasons. First, overproduction of hCdk4 mutants in which the inhibitory tyrosine is changed to phenylalanine abrogates G_1 arrest in response to ultraviolet (UV) light (*16*). Second, the UV sensitivity of *chk1*$^-$ mutants in *S. pombe* is suppressed by inactivating *cdc25* with a temperaturesensitive mutation (*10*). Finally, in *S. pombe wee1 mik1* mutants, DNA damage still causes a partial cell cycle delay that could be due to regulation of spCdc25 activity (*12*). GST-hChk1 and GST-hChk1(D130A) were introduced into baculovirus, purified from baculovirus-infected insect cells, and incubated with GST-hCdc25A, GST-hCdc25B, and GST-hCdc25C (*9*, *17*). GST-hChk1 phosphorylated all three hCdc25 proteins but not GST alone (Fig. 4A). Although GST-hCdc25C comigrated with GST-hChk1, which autophosphorylates, increased phosphorylation was observed at that position

relative to phosphorylation in the presence of kinase alone, and phosphorylation of a GST-hCdc25C breakdown product was visible. In separate experiments with a His_6-tagged hChk1 derivative, there was phosphorylation of GST-hCdc25C (Fig. 4B). A catalytically inactive mutant failed to phosphorylate itself or any of the hCdc25 proteins (Fig. 4A).

Protein kinases often form complexes with their substrates. To see if this was the case for hChk1 and the Cdc25 proteins, GST-hCdc25 proteins on glutathione beads were incubated together with baculovirus extracts expressing His_6-tagged hChk1 and precipitated. GST-hCdc25A, GST-hCdc25B, and GST-hCdc25C each specifically bound hChk1 whereas GST alone did not (Fig. 4C). Furthermore, two other GST fusion proteins, GST-Dun1 and GST-Skp1, failed to bind hChk1 (*18*). These results indicate that Cdc25 can form complexes with hChk1.

To establish the significance of the Cdc25 phosphorylation, we mapped the site of hChk1 phosphorylation on Cdc25C. The Ser^{216} residue is the main site of phosphorylation of hCdc25C in vivo (*19*). hChk1 phosphorylated a 56–amino acid region of the hCdc25C protein fused to GST (*19*) but not GST alone (Fig. 4A). This 56–amino acid motif contains four possible sites of phosphorylation. Peptide analysis of proteolytic fragments of full-length His_6-hCdc25C phosphorylated by GST-hChk1 revealed a single phosphorylated tryptic peptide by HPLC. Edman degradation of this peptide indicated release of radioactivity in the third cycle (Fig. 4D). Further degradation of this tryptic fragment with proline endopeptidase resulted in a peptide that released radioactivity in the first cycle (*17*). The Ser^{216} residue is the only site on hCdc25C consistent with this phosphorylation pattern (Fig. 4D). To confirm this, we constructed the Cdc25C S216A mutation in GST-Cdc25C and Cdc25C(200–256). Both were poor substrates for hChk1, confirming Ser^{216} as the site of phosphorylation (Fig. 4C). Serine-216 is also phosphorylated by spChk1, demonstrating phylogenetic conservation of this regulatory relation (*20*).

We have shown that the Chk1 kinase family is conserved throughout eukaryotic evolution and that hChk1, like its *S. pombe* counterpart, is modified in response to DNA damage. This, together with the fact that ATM-related kinases are conserved members of checkpoint pathways and act upstream of *chk1* in *S. pombe*, suggests that this entire checkpoint pathway may be conserved in all eukaryotes. hChk1 directly phosphorylates a regulator of Cdc2 tyrosine phosphorylation, hCdc25C, on a physiologically significant residue, Ser^{216}. Support for this comes from the work of Peng *et al.* (*20*) who have shown that the same site, which is the major site of Cdc25C phosphorylation during interphase, binds 14-3-3 proteins when phosphorylated and acts in an inhibitory fashion on hCdc25C. Overexpression of the hCdc25C S216A protein reduces the ability of cells to arrest in G_2 in response to DNA damage as observed previously for the Cdc2AF mutants (*12*). The overexpression studies alone do not prove that the DNA damage checkpoint pathway operates through tyrosine phosphorylation, because hyperactive Cdc2 may be able to bypass checkpoint control. However, in combination with the fact that this inhibitory serine is directly phosphorylated by the DNA damage–responsive checkpoint kinase hChk1, these results strongly imply that DNA damage regulates the G_2-to-mitosis transition through control of Cdc2 tyrosine phosphorylation. These results suggest a model whereby in response to DNA damage, hChk1 phosphorylates hCdc25C on Ser^{216}, which leads to binding of 14-3-3 protein and inhibition of Cdc25C's ability to dephosphorylate and activate Cdc2, a model that will require genetic verification.

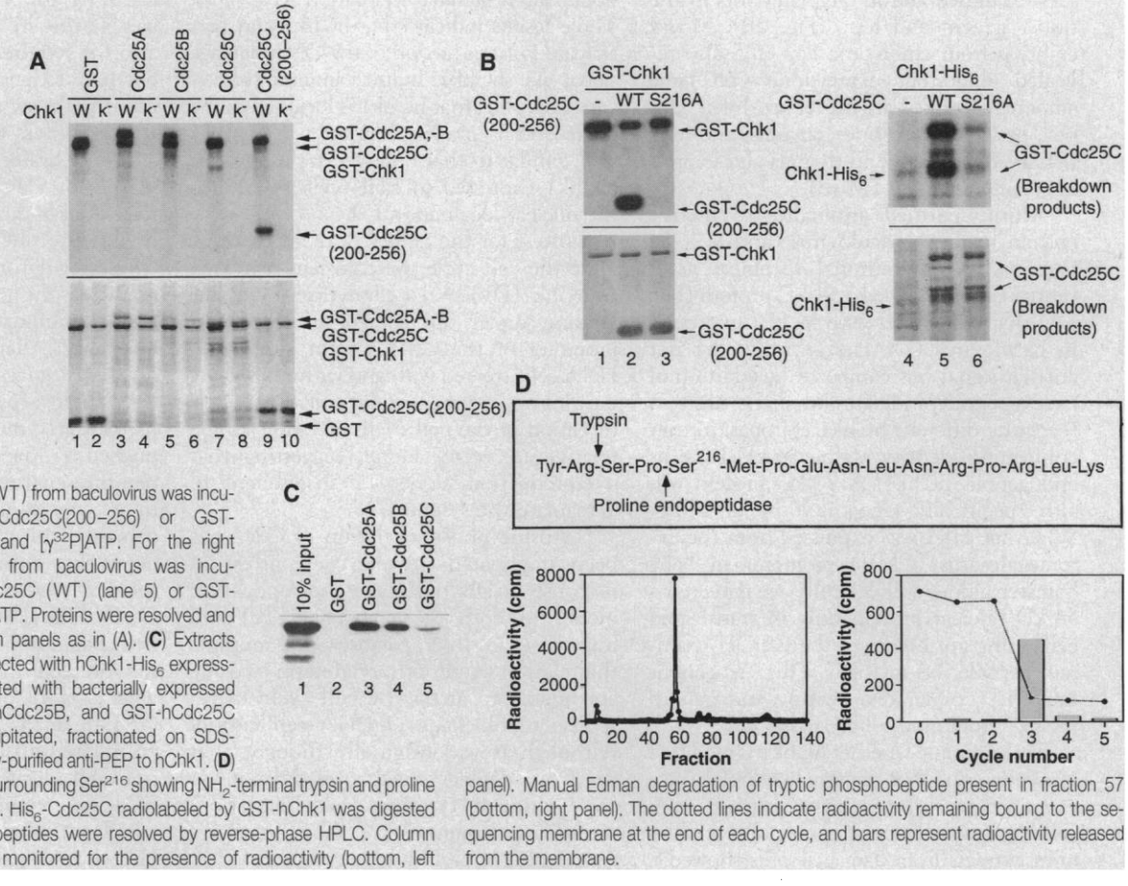

Fig. 4. hChk1 binds to and phosphorylates hCdc25A, hCdc25B, and hCdc25C. (**A**) GST-hChk1 (W) and GST-hChk1(D130A) (k^-) were purified from baculovirus and incubated with either GST, GST-hCdc25A, GST-hCdc25B, GST-hCdc25C, or GST-Cdc25C(200–256) and [γ^{32}P]ATP. Proteins were resolved by SDS-PAGE (10%) and visualized by autoradiography (for the kinase assay, top) or Coomassie blue staining (bottom). Less GST-Cdc25B was loaded than the other substrates. GST-hChk1 did not phosphorylate GST alone. (**B**) For the left panels, GST-hChk1 (WT) from baculovirus was incubated with either GST-hCdc25C(200–256) or GST-hCdc25C(200–256)(S216A), and [γ^{32}P]ATP. For the right panels, hChk1-His_6 purified from baculovirus was incubated with either GST-hCdc25C (WT) (lane 5) or GST-hCdc25C(S216A) and [γ^{32}P]ATP. Proteins were resolved and visualized for top and bottom panels as in (A). (**C**) Extracts prepared from insect cells infected with hChk1-His_6 expressing baculovirus were incubated with bacterially expressed GST, GST-hCdc25A, GST-hCdc25B, and GST-hCdc25C bound to GSH beads, precipitated, fractionated on SDS-PAGE, and probed with affinity-purified anti-PEP to hChk1. (**D**) Amino acids inclusive of and surrounding Ser^{216} showing NH_2-terminal trypsin and proline endopeptidase cleavage sites. His_6-Cdc25C radiolabeled by GST-hChk1 was digested with trypsin, and the tryptic peptides were resolved by reverse-phase HPLC. Column fractions were collected and monitored for the presence of radioactivity (bottom, left panel). Manual Edman degradation of tryptic phosphopeptide present in fraction 57 (bottom, right panel). The dotted lines indicate radioactivity remaining bound to the sequencing membrane at the end of each cycle, and bars represent radioactivity released from the membrane.

This model does not preclude a role for other cell cycle regulators such as Wee1 in the damage response (*14*). Furthermore, the fact that hChk1 phosphorylated hCdc25A and hCdc25B and that Ser^{216} is conserved among these Cdc25 proteins (*19*) suggests that hChk1 may regulate other DNA damage checkpoints, such as those controlling the G_1-to-S phase transition, through a similar mechanism.

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3. Degenerate primers GGNGGNGAGT/CT/CT-NATGGAT/CTT and TGGACAGGCCAAAGTC to conserved motifs in the kinase domains of spChk1 were used to screen a human B cell library by PCR. Four of 135 clones showed similarity to spChk1, and one was used to probe 2×10^5 plaques from a λACT human B cell cDNA library. We identified two *CHK1* cDNAs.
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9. We made recombinant baculovirus encoding glutathione-S-transferase (GST) fusion proteins to hChk1 (GST-hChk1) or to a mutation of hChk1 in which Asp at position 130 was mutated to Ala [GST-hChk1(D130A)] (pYS71). These recombinants were made by introducing an Nde I site at the first ATG codon of the hChk1 open reading frame using PCR, and subcloning the hChk1 cDNA as an Nde I–Eco RI fragment into pGEX2Tcs, generating pYS45. The Xba I–Eco RI fragment from pYS45 containing GST-hChk1 was subcloned into PVL1393, which was cut with Xba I and Eco RI, generating pYS63. The GST-hChk1(D130A) mutant was generated by PCR, and the Xho I–Xmn I fragment containing the mutation was used to replace the wild-type fragment, generating pYS64. The GST-hChk1(D130A) fragment from pYS64 was subloned into the baculovirus transfer vector by using the Univector plasmid fusion strategy (X. Liu and S. Elledge, unpublished results). Viruses were generated by standard methods (Baculogold, Pharmingen). Recombinant GST-hChk1 protein was isolated from infected Hi5 insect cells on glutathione (GSH) agarose. Cdc25C was cloned into pET15b (Novagen) and purified as outlined by the manufacturer.
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17. Kinase reactions contained GST-hChk1 bound to GSH agarose and either His_6-hCdc25C GST-hCdc25A, GST-hCdc25B, GST-hCdc25C, or GST-hCdc25C-(200–256) (amino acids 200 to 256 of Cdc25). Kinase reactions contained 1 to 3 μg of GST-hChk1 or GST-hChk1(D130A) protein on beads and soluble substrate in 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 μM adenosine triphosphate (ATP), and 15 μCi of [γ-^{32}P]ATP for 30 min at 30°C. To determine the site on Cdc25C phosphorylated by hChk1, we carried out kinase reactions in a buffer consisting of 50 mM tris (pH 7.4), 10 mM $MgCl_2$, 10 μM ATP, 1 mM dithiothreitol (DTT), and 10 μCi of [γ-^{32}P]ATP. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and visualized by autoradiography. The nitrocellulose membrane containing His_6-Cdc25C was excised, blocked with 0.5% polyvinylpyrrolidone (PVP-40) in 100 mM acetic acid for 30 min at 37°C, washed six times with water, and digested with TPCK trypsin (Worthington) at a final concentration of 30 mg/ml in 0.1 M NH_4CO_3 (pH 8.0). Further digestion on selected high-pressure liquid chromatography (HPLC) fractions was performed with 2 units of proline-specific endopeptidase (ICN) in 0.1 M sodium phosphate, 5 mM EDTA (pH 7.4) at 37°C for 16 hours. Samples were acidified in 1% trifluoroacetic acid (TFA) and loaded onto a Vydac C18 column (25 cm by 0.46 cm inner diameter). Reverse-phase HPLC was performed at 37°C. Reactions were loaded in 0.1% TFA (buffer A) and eluted with a gradient from 0 to 60% buffer B (90% acetonitrile, 0.095% TFA). Fractions were collected at 0.5-min intervals up to 90 min and counted for radioactivity. Selected fractions were immobilized on Sequenlon-AA membrane discs (Millipore) for NH_2-terminal sequencing. Manual Edman degradation was done as described (*21*) with a coupling and cleavage temperature of 55°C.
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Mitotic and G_2 Checkpoint Control: Regulation of 14-3-3 Protein Binding by Phosphorylation of Cdc25C on Serine-216

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Human Cdc25C is a dual-specificity protein phosphatase that controls entry into mitosis by dephosphorylating the protein kinase Cdc2. Throughout interphase, but not in mitosis, Cdc25C was phosphorylated on serine-216 and bound to members of the highly conserved and ubiquitously expressed family of 14-3-3 proteins. A mutation preventing phosphorylation of serine-216 abrogated 14-3-3 binding. Conditional overexpression of this mutant perturbed mitotic timing and allowed cells to escape the G_2 checkpoint arrest induced by either unreplicated DNA or radiation-induced damage. Chk1, a fission yeast kinase involved in the DNA damage checkpoint response, phosphorylated Cdc25C in vitro on serine-216. These results indicate that serine-216 phosphorylation and 14-3-3 binding negatively regulate Cdc25C and identify Cdc25C as a potential target of checkpoint control in human cells.

A key step in regulating the entry of eukaryotic cells into mitosis is the activation of the protein kinase Cdc2 by the protein phosphatase Cdc25C. A complete understanding of mitotic control requires elucidation of the mechanisms that regulate the interactions between Cdc2 and Cdc25C throughout the cell cycle. Furthermore, although tremendous progress has been made in recent years in identifying proteins that participate in checkpoint control, it is unclear how these proteins interface with core cell cycle regulators to inhibit cell cycle transitions (*1*).

The Ser^{216} residue is the primary site of phosphorylation of Cdc25C in asynchronously growing cells (*2*). To determine if phosphorylation of Ser^{216} regulates Cdc25C function, we generated HeLa cell lines that allow conditional expression of either wild-type Cdc25C or a mutant of Cdc25C containing alanine at position 216 (S216A). In these cells, expression of Cdc25C and Cdc25(S216A) is under the control of a

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