

Differential Mode of Regulation of the Checkpoint Kinases CHK1 and CHK2 by Their Regulatory Domains*

Received for publication, November 7, 2003, and in revised form, December 16, 2003
Published, JBC Papers in Press, December 16, 2003, DOI 10.1074/jbc.M312215200

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CHK1 and CHK2 are key mediators that link the machineries that monitor DNA integrity to components of the cell cycle engine. Despite the similarity and potential redundancy in their functions, CHK1 and CHK2 are unrelated protein kinases, each having a distinctive regulatory domain. Here we compare how the regulatory domains of human CHK1 and CHK2 modulate the respective kinase activities. Recombinant CHK1 has only low basal activity when expressed in cultured cells. Surprisingly, disruption of the C-terminal regulatory domain activates CHK1 even in the absence of stress. Unlike the full-length protein, C-terminally truncated CHK1 displays autophosphorylation, phosphorylates CDC25C on Ser²¹⁶, and delays cell cycle progression. Intriguingly, enzymatic activity decreases when the entire regulatory domain is removed, suggesting that the regulatory domain contains both inhibitory and stimulatory elements. Conversely, the kinase domain suppresses Ser³⁴⁵ phosphorylation, a major ATM/ATR phosphorylation site in the regulatory domain. In marked contrast, CHK2 expressed in either mammalian cells or in bacteria is already active as a kinase against itself and CDC25C and can delay cell cycle progression. Unlike CHK1, disruption of the regulatory domain of CHK2 abolishes its kinase activity. Moreover, the regulatory domain of CHK2, but not that of CHK1, can oligomerize. Finally, CHK1 but not CHK2 is phosphorylated during the spindle assembly checkpoint, which correlates with the inhibition of the kinase. The mitotic phosphorylation of CHK1 requires the regulatory domain, does not involve Ser³⁴⁵, and is independent on ATM. Collectively, these data reveal the very different mode of regulation between CHK1 and CHK2.

In mammalian cells, DNA damage checkpoints operate throughout the cell cycle to monitor and maintain genomic stability. These checkpoints ensure that damaged DNA is not replicated in S phase or segregated to the daughter cells in mitosis until repaired. Cells that harbor defects in these pathways are prone to genomic instability and neoplastic transformation.

* This work was supported in part by Research Grants Council Grants HKUST6129/02M (to R. Y. C. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The gene mutated in ataxia-telangiectasia (AT),¹ *ATM*, encodes one of the critical protein kinases activated by DNA double strand breaks (1). Cells derived from AT patients exhibit a greatly enhanced radiosensitivity, radioresistant DNA synthesis, and chromosomal instability. After ionizing radiation (IR)-induced DNA damage, ATM phosphorylates and activates CHK2, which in turn phosphorylates CDC25C on Ser²¹⁶ (2). This either directly inactivates the phosphatase activity of CDC25C or indirectly inactivates it through the creation of a 14-3-3 binding site (3, 4). IR-induced destruction of another member of the CDC25 family, CDC25A, requires CHK2-mediated phosphorylation of CDC25A on Ser¹²³ (5, 6). CDC25A and CDC25C activate CDC2 by dephosphorylating two residues (Thr¹⁴ and Tyr¹⁵) located within the catalytic cleft. Thus, inactivation of CDC25 promotes inhibitory phosphorylation and inactivation of CDC2. This mechanism is critical for the G₂ DNA damage checkpoint, because the cyclin B1-CDC2 complex is a key regulator of mitotic entry (7). Similarly, the ATM-CHK2-CDC25A pathway is also essential for the intra-S phase DNA damage checkpoint by inhibiting CDK2 (6).

In addition to acting through the CHK2-CDC25A-CDK pathways, ATM also acts on a parallel pathway involving the trimeric protein complex MRE11-NBS1-RAD50 (8). In response to IR, the forkhead-associated (FHA) domain-containing protein MDC1 is phosphorylated in an ATM-dependent manner, relocalizes to nuclear foci containing the MRE11-NBS1-RAD50 complex, and associates with CHK2 (9–11).

The ATM- and Rad3p-related kinase ATR is activated by a broader spectrum of genotoxic stimuli than ATM (12, 13). These include IR, UV, and other agents that induce DNA cross-linking as well as inhibitors of DNA replication. Several lines of evidence suggest that whereas the effects of ATM are mediated mainly through CHK2, the effects of ATR are mediated through another kinase called CHK1. Disruption of *CHK1* in mice results in peri-implantation embryonic lethality (14, 15). In contrast, *CHK2*^{-/-} mice develop normally and do not develop tumors spontaneously (16, 17). In response to DNA damage or replication block, CHK1 is phosphorylated at Ser³¹⁷ and Ser³⁴⁵ by ATR (14, 18). Like CHK2, CHK1 also phosphorylates and inactivates CDC25 (19–21). In addition, CHK1 also phosphorylates WEE1, the protein kinase that opposes the action of CDC25 by phosphorylation of CDC2 on Tyr¹⁵ (22); this creates a 14-3-3 binding site and stimulates the kinase activity of WEE1 (23).

As the mediators that link the DNA stress responses to the cell cycle, CHK1 and CHK2 share many similarities in func-

¹ The abbreviations used are: AT, ataxia-telangiectasia; IR, ionizing radiation; FHA, forkhead-associated; HU, hydroxyurea; NOC, nocodazole; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein.

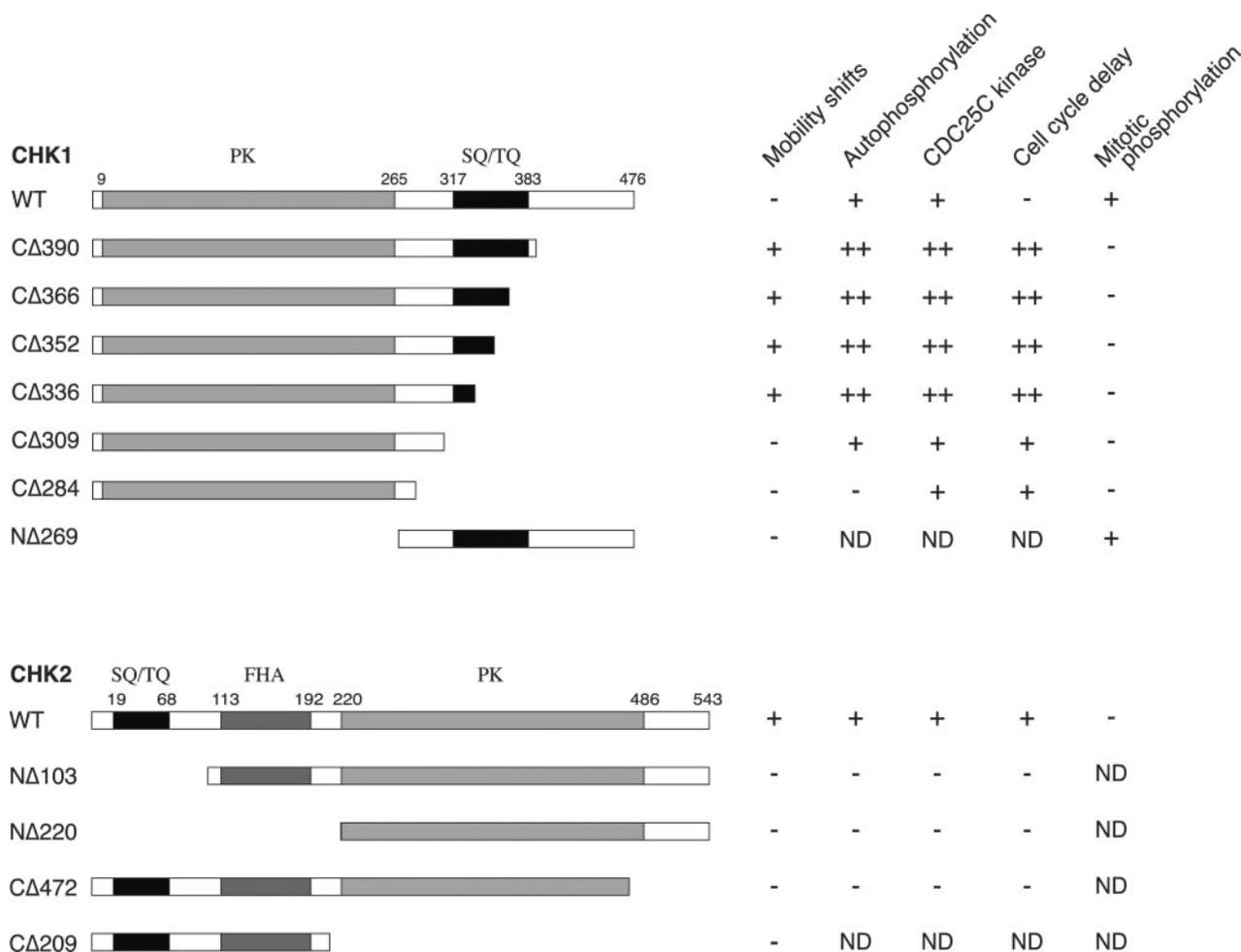


FIG. 1. Schematic diagram of the CHK1 and CHK2 constructs used in this study. The positions of the various structural elements (protein kinase domain (PK), SQ/TQ domain, and FHA domain) are shown to scale. The mobility shifts on SDS-PAGE, autophosphorylation and kinase activity against GST-CDC25C₁₉₅₋₂₅₉ after immunoprecipitation, and the NOC-induced mobility shifts of the kinase-inactive version of the constructs are summarized (see "Results" for details). ND, not determined.

tions as well as regulation. However, these two protein kinases share no significant sequence similarity outside the protein kinase domain. CHK1 comprises a kinase domain at the N-terminal half and a regulatory domain at the C-terminal half of the protein. An SQ/TQ domain (residues 317–383) contains putative phosphorylation sites for ATM/ATR and lies within the regulatory domain. CHK1 is phosphorylated on Ser³¹⁷ and Ser³⁴⁵ in response to replication stress or other types of DNA damage in an ATM/ATR-dependent fashion (14, 18, 24–26). Other checkpoint components including the Hus1p of the 9-1-1 complex (27) and Rad17p (28) also act upstream of CHK1 phosphorylation. Another upstream regulator of CHK1, claspin, is phosphorylated and binds to CHK1 after replication block or DNA damage. Phosphorylated claspin docks with a phosphate-binding site in the kinase domain of CHK1, which may mimic activating phosphorylation (29–32).

In comparison with CHK1, the kinase domain of CHK2 is located at the C-terminal half of the protein. The N-terminal regulatory region contains an SQ/TQ domain (residues 19–68) and an FHA domain. Thr⁶⁸ in the SQ/TQ domain is a major ATM phosphorylation site (33–36). Other sites are also phosphorylated by ATM, because only mutation of all seven SQ/TQ sites abolishes phosphorylation (35). The FHA domain is dispensable for Thr⁶⁸ phosphorylation but necessary for efficient autophosphorylation in response to IR (37–39). After DNA damage, Thr⁶⁸ phosphorylation triggers autophosphorylation of Thr³⁸³ and Thr³⁸⁷ (40). These residues are located within the activating loop of the kinase domain and are essential for

CHK2 activity. The FHA domain also mediates CHK2 oligomerization via direct interaction with phosphorylated Thr⁶⁸ on another CHK2 molecule (37, 38). Although Thr⁶⁸ phosphorylation may be required for initial oligomerization and activation of CHK2, it is not needed for maintenance of oligomerization or kinase activity (41).

Many protein kinases possess an integral regulatory domain that mediates a variety of functions including interactions with other proteins, activation, or inhibition of the kinase domain (42). The precise functions of the regulatory domains of CHK1 and CHK2 remain to be deciphered. Here we address the issue by creating a series of truncation mutants of human CHK1 and CHK2. We found that full-length CHK1 has only low basal activity when expressed in mammalian cells. Surprisingly, disruption of the regulatory domain activates CHK1 even in the absence of stress. In contrast, ectopically expressed full-length CHK2 is already active as a kinase and can inhibit the cell cycle. Unlike CHK1, disruption of the regulatory domain of CHK2 abolishes its kinase activity. Moreover, the regulatory domain of CHK2, but not that of CHK1, can form oligomers. Finally, the regulatory domain of CHK1 is phosphorylated during mitotic block, which correlates with the inhibition of CHK1 kinase activity. Collectively, these data reveal the very different mode of regulation between CHK1 and CHK2.

EXPERIMENTAL PROCEDURES

Materials—All reagents were obtained from Sigma unless stated otherwise.

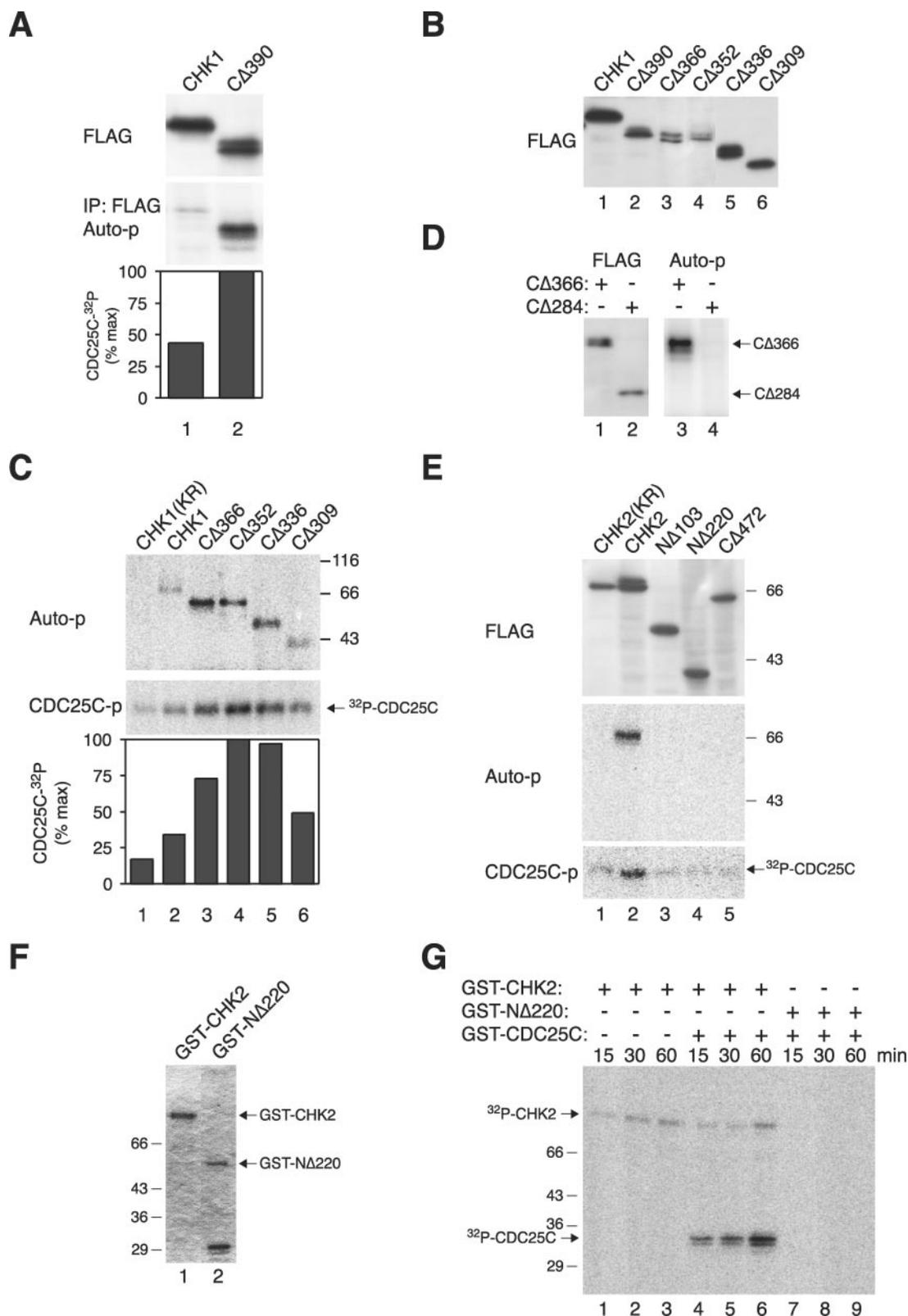


FIG. 2. The regulatory domains of CHK1 and CHK2 have opposite effects on the basal kinase activity. *A*, C-terminal truncation activates CHK1. HtTA1 cells were transfected with plasmids expressing FLAG-tagged CHK1 or CΔ390 as indicated. At 24 h after transfection, cell extracts were prepared, and the FLAG-tagged proteins were detected by immunoblotting (*top panel*). Immunoprecipitates (*IP*) of FLAG antiserum were assayed for autophosphorylation (*Auto-p*; *middle panel*) and kinase activity against GST-CDC25C₁₉₅₋₂₅₉ (*bottom panel*) as described under "Experimental Procedures." *B*, gel mobility shifts of recombinant CHK1. FLAG-CHK1 and the indicated truncation mutants were expressed in HtTA1 cells. At 24 h after transfection, cell extracts were prepared, and the FLAG-tagged proteins were detected by immunoblotting. *C*, kinase activity of recombinant CHK1. FLAG-tagged CHK1, CHK1(K38R), and the indicated truncation mutants were expressed in HtTA1 cells. At 24 h after transfection, cell extracts were prepared, and the FLAG-tagged proteins were immunoprecipitated with FLAG antiserum. The autophosphorylation and kinase activity against GST-CDC25C₁₉₅₋₂₅₉ were assayed. The positions of molecular size standards (in kDa) are shown on the *right*. The phosphorylation of the recombinant CDC25C was quantified with a PhosphorImager and is shown in the *bottom panel*. *D*, CHK1 lacking the entire regulatory domain has no autophosphorylation activity. FLAG-CHK1(CΔ366) and FLAG-CHK1(CΔ284) were expressed in HtTA1 as indicated. Cell extracts were prepared and the samples were subjected to immunoblotting for FLAG-tagged proteins or to an autophosphorylation

DNA Constructs—All clones in this study were from human origin. GST-CDC25C_{195–259} in pGEX-KG (containing residues 195–259 of CDC25C) and cDNA for CHK1 and CHK2 were gifts from Dr. Katsumi Yamashita (Kanazawa University, Japan). CHK1 was amplified by PCR with primers 5'-TGAATTCATGGCAGTGCCTTTGTGG-3' (CHK1-FOR) and 5'-GGAATTCATGTGGCAGGAAGCCA-3' (CHK1-REV); the PCR product was cut with EcoRI and ligated into pUHD-P1 (43) to create FLAG-CHK1 in pUHD-P1. CHK1 cDNA was amplified by PCR with CHK1-FOR and 5'-GGAATTCCTAAGATTTGTCTGCATC-3' (CA390), 5'-CGAATTCCTGAAATGTATGAGGGGCT-3' (CA336), 5'-CGAATTCCTGAACTAGAAGCACTGTT-3' (CA309), or 5'-GGAATTCCTCACACACCACCTGAAGTGACTCGG-3' (CA284); the PCR products were cut with EcoRI and ligated into pUHD-P1 to obtain the respective truncation mutants. FLAG-CHK1 in pUHD-P1 was cut with BamHI and religated to obtain CA366 or cut with BclI-BamHI and religated to create CA352. CHK1 was amplified by PCR with 5'-GGAATTCATGGCAGTGCCTTTGTGG-3' and CHK1-REV, cut with EcoRI, and ligated into pUHD-P2 (44) to create HA-CHK1 in pUHD-P2. The product from PCR amplification with primer 5'-CACCATGGGGGCAAAAAGGCCCG-3' and a vector reverse primer was cut with NcoI and ligated into pUHD-P2 to create HA-CHK1(NΔ269) in pUHD-P2. CHK2 cDNA was amplified by PCR with primers 5'-AGAATTCATGTCTCGGGAGTCCGATG-3' and 5'-AGAATTCCTACAACACAGCAGCACA-3' (CHK2-REV); the PCR product was cut with EcoRI and ligated into pUHD-P1 and pGEX-6P-1 (Amersham Biosciences) to create FLAG-CHK2 in pUHD-P1 and GST-CHK2 in pGEX-6P-1, respectively. CHK2 cDNA was amplified by PCR with CHK2-REV and 5'-TGAATTCGCCAATCTTGAAATGTGTGA-3' (NΔ103) or 5'-TGAATTCATCATGTCAAAAATC-3' (NΔ220); the PCR products were cut with EcoRI and ligated into pUHD-P1 to obtain the respective truncation mutants. The NΔ220 fragment was also ligated into pGEX-6P-1 to create GST-CHK2(NΔ220) in pGEX-6P-1. The BamHI-BamHI fragment was released from FLAG-CHK2 in pUHD-P1 to create the CA472 mutant. CHK2 was amplified by PCR with 5'-GGAATTCATGTCTCGGGAGTCCGG-3' and CHK2-REV, cut with EcoRI, and ligated into pUHD-P2 (44) to create HA-CHK2 in pUHD-P2. The BclI-BamHI fragment was released to create HA-CHK2(CA209) in pUHD-P2. Site-directed mutagenesis was carried out by a PCR method (45), using the oligonucleotides 5'-GTCGAGTGAGGATTGTAGATA-3' (for CHK1(K38R)), 5'-GTAGCCATAAGGATCATCAGCAA-3' (for CHK2(K249R)), and their antisense to introduce the mutations.

Cell Culture—HtTA1 cells are HeLa cells (cervical carcinoma) expressing the tTA tetracycline repressor chimera (44). Normal human lymphoblastoid cells (GM03798) and AT lymphoblastoid cells (GM03189) were obtained from Coriell Cell Repositories (Camden, NJ). HL-60 (acute promyelocytic leukemia) and Ramos (Burkitt's lymphoma) were obtained from the American Type Culture Collection (Manassas, VA). Unless stated otherwise, cells were treated with the following reagents at the indicated final concentration: adriamycin (200 ng/ml), aphidicolin (5 μg/ml), caffeine (5 mM), camptothecin (0.67 μM), cisplatin (2 μg/ml), etoposide (10 μg/ml), hydroxyurea (HU) (1.5 mM), lovastatin (20 μM), nocodazole (NOC) (0.1 μg/ml), taxol (0.5 μg/ml), thymidine (2 mM), tumor necrosis factor α (100 units/ml), and TRAIL (100 ng/ml). Cells were transfected with a calcium phosphate precipitation method (46), and cell-free extracts were prepared as described (47). Cell cycle synchronization by double thymidine block (48) or NOC block (49) was as described previously.

Flow Cytometry—Flow cytometry analysis after propidium iodide staining were performed as described (50). In some experiments, transfected cells were distinguished from nontransfected cells by co-transfection of a membrane-targeted GFP reporter (51) (a gift from Drs. Wai Jiang and Tony Hunter, The Salk Institute, La Jolla, CA).

Kinase Assays and Phosphatase Treatments—Expression of recombinant GST-CDC25C_{195–259} in bacteria and purification with GSH-agarose chromatography were as described (47). Immunoprecipitates equilibrated with kinase buffer (80 mM Na-β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM Mg(OAc)₂, and 1 mM dithiothreitol) were mixed with 10 μl of

kinase buffer containing 50 μM ATP, 1.25 μCi of γ-³²PATP, and 1 μg of GST-CDC25C_{195–259}. The samples were incubated at 23 °C for 30 min, and the reactions were terminated by the addition of 30 μl of SDS-sample buffer. The samples were applied onto SDS-PAGE, and phosphorylation was quantified with a PhosphorImager (Amersham Biosciences). For dephosphorylation reactions, immunoprecipitates were treated with 1 unit of calf intestine alkaline phosphatase (Promega, Madison, WI) in 10 μl of reaction mix at 37 °C for 30 min. The phosphatase was inhibited by 10 mM β-glycerophosphate and 10 mM NaF.

Antibodies and Immunological Methods—Immunoblotting and immunoprecipitation were performed as described (47). Rabbit polyclonal antibodies against FLAG tag (44), monoclonal antibody A17 against CDC2 (52), E23 against cyclin A2 (52), YL1/2 against tubulin (52), 12CA5 against HA tag (53), and M2 against FLAG tag (53) were obtained from sources as described previously. CHK1 antiserum was a generous gift from Dr. Helen Piwnicka-Worms (Washington University, St. Louis, MO). Fas antiserum was a gift from Dr. Benjamin Bonavida (UCLA, Los Angeles, CA). Antibodies against Ser³⁴⁵-phosphorylated CHK1 and Tyr¹⁵-phosphorylated CDC2 were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies against CHK1 (sc-8408), CHK2 (sc-5278 and sc-17747), cyclin B1 (sc-245), and cyclin E (sc-247) and polyclonal antibodies against Ser¹⁰-phosphorylated histone H3 (sc-8656R) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

RESULTS

The Regulatory Domain Suppresses the Kinase Activity of CHK1—This study was initiated by our observations that CHK1 was significantly overexpressed in liver cancer (hepatocellular carcinoma) in comparison with nontumorous liver tissues from the same patients.² Given that the overexpressed CHK1 apparently does not hinder liver cancer cell growth, we hypothesized that full-length CHK1 may not be active even when overexpressed. To see whether recombinant CHK1 is active, both full-length CHK1 and a mutant lacking part of the C terminus (CA390; see Fig. 1 for schematic diagram) were ectopically expressed in mammalian cells (Fig. 2A). FLAG epitope tags were introduced to distinguish the recombinant proteins from the endogenous CHK1. We found that whereas full-length CHK1 appeared as a single band on SDS-PAGE, CA390 exhibited mobility shifts that are typical of phosphorylation. Consistent with the mobility shifts, CHK1 was only weakly radiolabeled, but CA390 was robustly autophosphorylated when incubated with radioactive ATP. To measure the kinase activity of CHK1 against external substrates, a GST-CDC25C_{195–259} fusion protein containing the CHK1 phosphorylation site (Ser²¹⁶) was used. Consistent with the autophosphorylation activity, CDC25C was phosphorylated by CA390 more strongly than by full-length CHK1. Phosphorylation was specific for Ser²¹⁶, because a GST-CDC25C_{195–259} with Ser²¹⁶ mutated to alanine was not phosphorylated by recombinant CHK1 (data not shown).

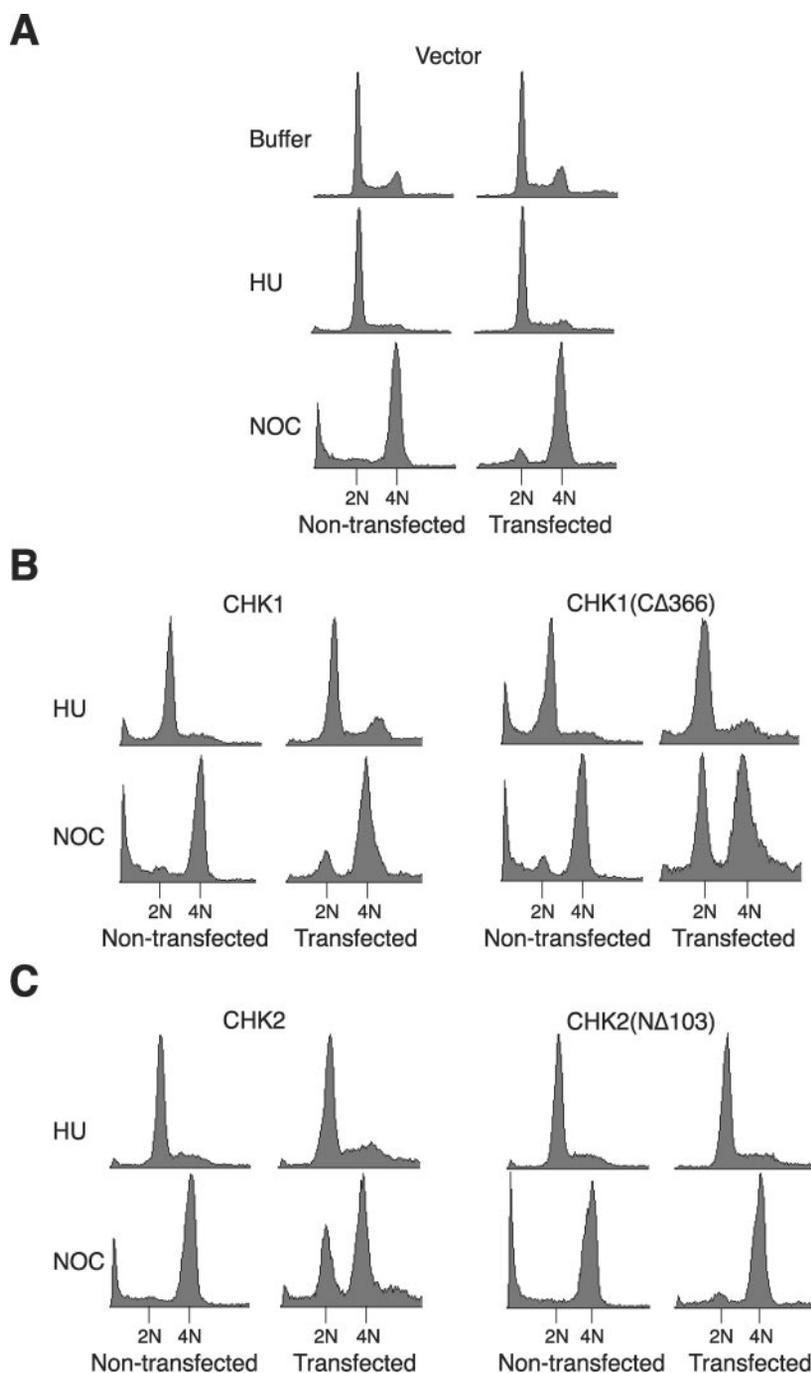
Given that CA390 but not full-length CHK1 displayed gel mobility shifts, we next characterized the regions involved in the mobility shifts with further truncations. Fig. 2B shows that progressive truncation of CHK1 from the C terminus did not abolish the mobility shifts until the entire SQ/TQ domain was removed (CA309; see Fig. 1). No mobility shift was detected

² C.-P. Ng, H. C. Lee, C. W. Ho, T. Arooz, W. Y. Siu, A. Lau, and R. Y. C. Poon, unpublished data.

assay after immunoprecipitation with FLAG antiserum (lanes 3 and 4). *E*, disruption of the N-terminal regulatory domain of CHK2 abolishes kinase activity. FLAG-tagged CHK2, CHK2(K249R), and various truncation mutants were expressed in HtTA1 cells as indicated. At 24 h after transfection, cell extracts were prepared, and the FLAG-tagged proteins were detected by immunoblotting (top panel). The autophosphorylation (middle panel) and kinase activity against GST-CDC25C_{195–259} (bottom panel) were assayed as described under "Experimental Procedures." *F*, purification of CHK2 from bacteria. GST-CHK2 and GST-CHK2(NΔ220) were expressed in *E. coli* and purified with GSH column chromatography. The purified proteins were applied onto SDS-PAGE and visualized by Coomassie Blue staining. The positions of molecular size standards (in kDa) are shown on the left. *G*, bacterially expressed CHK2 but not NΔ220 is active. About 0.1 μg of GST-CHK2 or GST-CHK2(NΔ220) was incubated with [γ-³²P]ATP and in the presence or absence of GST-CDC25C_{195–259} as indicated. Samples were harvested at the indicated time points, and phosphorylations were detected by SDS-PAGE and phosphorimaging. The positions of molecular size standards (in kDa) are shown on the left.

FIG. 3. Transient overexpression of CHK2 but not CHK1 causes G₁ delay.

A, cell cycle analysis of transiently transfected cells with a GFP reporter. HtTA1 cells were transfected with a membrane-targeted GFP reporter. After 12 h, NOC, HU, or buffer was added, and cells were incubated for another 16 h before they were harvested for flow cytometry analysis. Cell cycle distribution was analyzed for GFP-positive (transfected) and GFP-negative (nontransfected) cells. The positions of 2N and 4N DNA content are indicated. **B**, cell cycle delay induced by CHK1(Δ 366) but not CHK1. The experiments were performed similarly to that in **A** except that FLAG-tagged CHK1 or Δ 366 was co-expressed with the GFP reporter. **C**, cell cycle delay induced by CHK2 but not CHK2(Δ 103). The experiments were performed similarly to that in **A** except that FLAG-tagged CHK2 or Δ 103 was co-expressed with the GFP reporter.



when the Lys residue critical for catalysis was mutated to Arg (K38R) (see below), indicating that it was mediated by autophosphorylation. Fig. 2C shows that CHK1 phosphorylated itself and GST-CDC25C₁₉₅₋₂₅₉ slightly above the background and the kinase-inactive CHK1(K38R). The autophosphorylation and kinase activities against CDC25C were in excellent correlation with the gel mobility shifts. We found that Δ 309 still displayed some autophosphorylation activity (Fig. 2C). Further deletion into the regulatory domain (Δ 284) completely abolished autophosphorylation (Fig. 2D). Taken together, these data show that CHK1 is activated (against itself and CDC25C) when part of the regulatory domain is deleted, suggesting that the regulatory domain may suppress the basal activity of CHK1. Interestingly, CHK1 is less active when the entire SQ/TQ domain is removed (see "Discussion").

To see whether the *in vitro* kinase activity of CHK1 correlated with its *in vivo* effects on the cell cycle, flow cytometry

analysis was performed after the expression of recombinant CHK1. A membrane-targeted GFP reporter was co-expressed to distinguish transfected and nontransfected cells. Fig. 3A shows that transfection of control vector did not significantly affect cell cycle distribution. More importantly, both transfected and nontransfected cells were trapped in early S phase and G₂/M by treatment with HU and NOC, respectively, indicating that the experimental procedure itself did not upset cell cycle progression. We found that whereas overexpression of CHK1 did not hinder cell cycle progression, expression of the Δ 366 severely compromised the NOC-induced G₂/M block (Fig. 3B). Similar expression of CHK1 and Δ 366 was confirmed by immunoblotting (data not shown). Since the HU-induced cell cycle block was unaffected, these results indicate that expression of the active Δ 366 delayed G₁ or S phase (it is not possible to distinguish between G₁ and S phase block with this type of analyses). We found that the G₁/S delay induced by

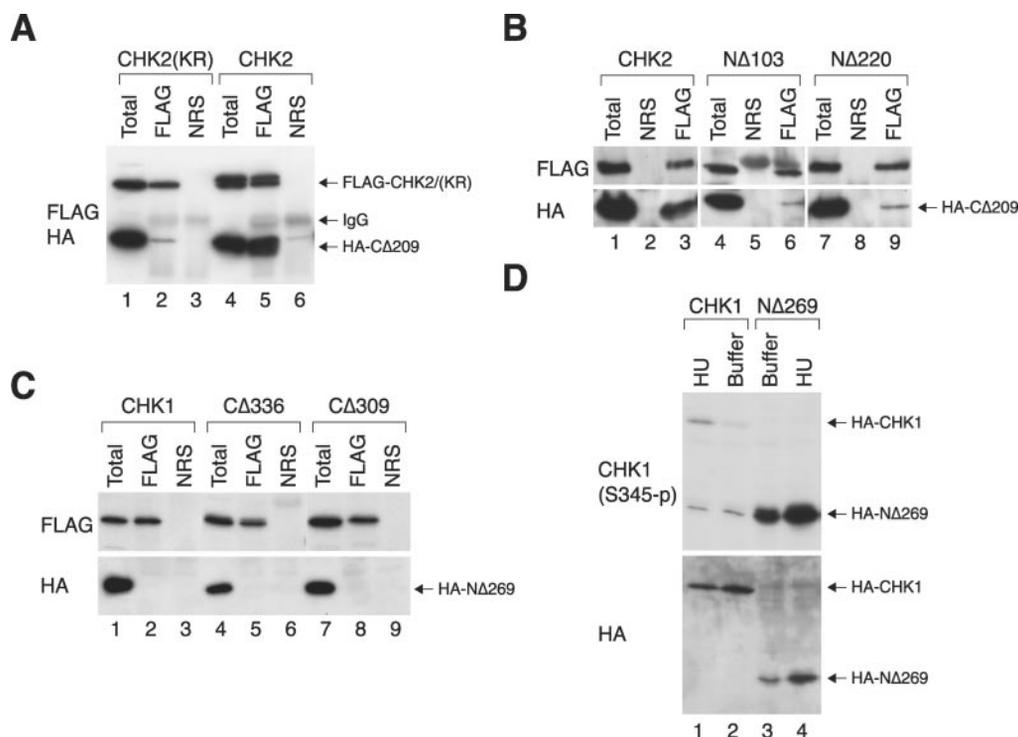


FIG. 4. Possible interactions between the regulatory domains and the kinase domains of CHK1 and CHK2. *A*, binding between full-length CHK2 and its regulatory domain requires active kinase. HA-CHK2(C Δ 209) was co-expressed with either FLAG-CHK2(K249R) or FLAG-CHK2 in HtTA1 cells. Cell extracts were prepared, and 100 μ g was subjected to immunoprecipitation with FLAG antiserum or normal rabbit serum control (NRS). Total cell lysates (10 μ g) and the immunoprecipitates were subjected to immunoblotting for FLAG and HA. *B*, the regulatory domain of CHK2 does not bind to the kinase domain. HA-CHK2(C Δ 209) was co-expressed with FLAG-tagged CHK2, N Δ 103, or N Δ 220, as indicated. Immunoprecipitation and immunoblotting were performed as in *A*. The fuzziest bands in lanes 5 and 6 are IgG. *C*, lack of direct interaction between the regulatory domain and kinase domain of CHK1. HA-CHK1(N Δ 269) was co-expressed with FLAG-tagged CHK1, C Δ 336, or C Δ 309 as indicated. Immunoprecipitation and immunoblotting were performed as in *A*. *D*, the kinase domain of CHK1 suppresses phosphorylation on Ser³⁴⁵. HA-CHK1 and HA-CHK1(N Δ 269) were expressed in HtTA1 cells. Cells were treated with either buffer or HU for 16 h. Cell extracts were prepared and subjected to immunoblotting with antibodies against Ser³⁴⁵-phosphorylated CHK1 and HA tag.

various truncation mutants of CHK1 correlated nicely with their *in vitro* kinase activity (summarized in Fig. 1). We conclude that deletion of the C-terminal region of CHK1 triggers gel mobility shifts, activates *in vitro* autophosphorylation and CDC25C kinase, and induces cell cycle arrest when overexpressed.

The Regulatory Domain of CHK2 Is Required for Its Kinase Activity—We next investigated whether the regulatory domain of CHK2 regulates its activity in a similar manner as CHK1. In marked contrast to CHK1, recombinant CHK2 displayed gel mobility shifts even in the absence of other stress (Fig. 2E). These mobility shifts of CHK2 were dependent on its own kinase activity, because shifts were absent with a kinase-inactive version of CHK2 (K249R) (lane 1). Surprisingly, truncation mutants lacking the SQ/TQ domain (N Δ 103) and FHA domain (N Δ 220) (see Fig. 1) abolished the gel mobility shifts. Unlike CHK1, full-length CHK2 displayed both autophosphorylation and kinase activity against CDC25C (Fig. 2E). As expected, K249R mutation or a mutant without part of the kinase domain (C Δ 472) lacked these activities. Consistent with the lack of mobility shifts, both mutants with disrupted regulatory domain were inactive.

GST fusion of CHK2 could be readily expressed and purified from bacteria (Fig. 2F). In agreement with published results (38, 54), purified GST-CHK2 phosphorylated itself and the GST-CDC25C_{195–259} substrate (Fig. 2G). In contrast, GST-CHK2(N Δ 220) did not autophosphorylate or phosphorylate CDC25C.

We found that expression of full-length CHK2 was sufficient to trigger a G₁/S cell cycle delay (Fig. 3C; summarized in Fig. 1). Consistent with the lack of *in vitro* kinase activity, expres-

sion of CHK2 lacking part of the regulatory domain (N Δ 103) did not influence cell cycle progression. Similar expression of CHK2 and N Δ 103 was confirmed by immunoblotting (data not shown). These data show that in contrast to CHK1, recombinant CHK2 is active as a kinase in the presence of the regulatory domain.

The Kinase Domain of CHK1 Suppresses Ser³⁴⁵ Phosphorylation but Does Not Physically Bind to the Regulatory Domain—The above results show that the regulatory domains of CHK1 and CHK2 modulate the respective kinase activity. We hypothesized that the regulatory domains may govern the kinase domains through direct intramolecular interactions, and deletion of the regulatory domains may impair these interactions. To test this hypothesis, the regulatory domain and the kinase domain were fused to different epitope tags and co-expressed. Fig. 4A shows that the regulatory domain of CHK2 (C Δ 209) was co-immunoprecipitated with full-length CHK2 (lane 5) but not with control serum immunoprecipitates (lane 6). In contrast, C Δ 209 only weakly associated with the kinase-inactive CHK2(K249R), suggesting that the binding required autophosphorylation. These results reproduced the conditions for phosphorylation-dependent binding between the regulatory domains of two molecules of CHK2 (37, 38). To test directly whether the regulatory domain of CHK2 can bind to its kinase domain, C Δ 209 was co-expressed with CHK2 lacking part or the entire regulatory domain (N Δ 103 and N Δ 220). Fig. 4B shows that whereas C Δ 209 efficiently bound to CHK2, very low levels of binding to N Δ 103 or N Δ 220 were detected. Similarly, no interaction was detected between the regulatory domain of CHK1 (N Δ 269) and its kinase domain (Fig. 4C). Moreover, N Δ 269

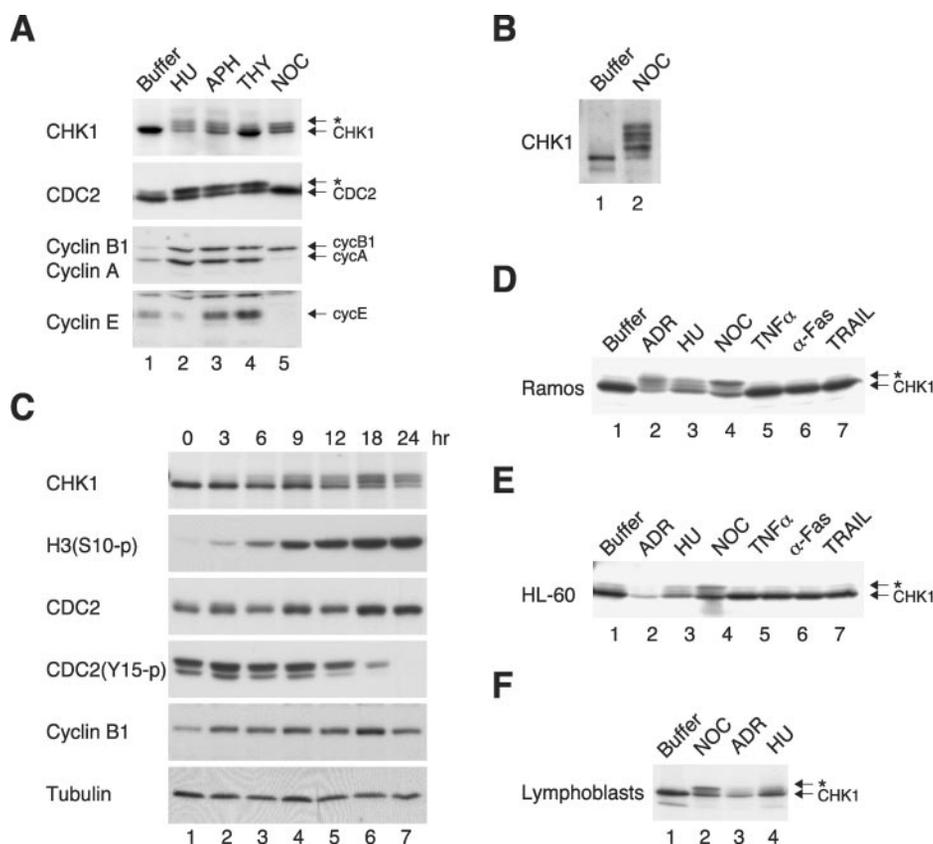


FIG. 5. CHK1 is phosphorylated during replication block and mitotic block. *A*, replication block and mitotic block triggers phosphorylation of CHK1. HtTA1 cells were treated with buffer, HU, aphidicolin, thymidine, or NOC for 16 h as indicated. Cell extracts were prepared and immunoblotted with antibodies against CHK1, CDC2, cyclin A, cyclin B1, and cyclin E. The asterisks indicate the positions of the slower migrating, phosphorylated forms of CHK1 and CDC2. *B*, extensive mobility shifts of CHK1 induced by mitotic block. HtTA1 cells were treated with either buffer or NOC for 16 h. Cell extracts were prepared, resolved on long SDS-PAGE, and subjected to immunoblotting for CHK1. *C*, temporal relationship between CHK1 phosphorylation and other mitotic markers following NOC treatment. NOC was added to HtTA1 cells, and cells were harvested at the indicated time points. Cell extracts were prepared and immunoblotted for CHK1, Ser¹⁰-phosphorylated histone H3 (*H3(S10-p)*), CDC2, Tyr¹⁵-phosphorylated CDC2 (*CDC2(Y15-p)*), and cyclin B1 as indicated. Equal loading of lysates was confirmed by immunoblotting for tubulin. *D*, mitotic phosphorylation of CHK1 in Ramos cells. Ramos cells were treated with buffer, adriamycin (ADR), HU, NOC, tumor necrosis factor α , anti-Fas antibodies, and TRAIL for 16 h as indicated. Cell extracts were prepared, and CHK1 was detected by immunoblotting. The asterisk indicates the position of the slower migrating, phosphorylated forms of CHK1. *E*, mitotic phosphorylation of CHK1 in HL-60 cells. The experiment was performed exactly as in *D* except that HL-60 cells were used. *F*, mitotic phosphorylation of CHK1 in lymphoblastoid cells. Wild type lymphoblastoid cells were treated with buffer, NOC, adriamycin, or HU as indicated. CHK1 was detected by immunoblotting.

did not bind full-length CHK1, suggesting that the type of dimer formation in CHK2 did not occur with CHK1. These data suggest that the influences of the regulatory domains of CHK1 and CHK2 on the kinases may be based on weak and transient interactions (see "Discussion").

To see whether the kinase domain of CHK1 has a reciprocal effect on the regulatory domain, we examined the status of Ser³⁴⁵ phosphorylation (a major stress-induced phosphorylation site in the regulatory domain; see Introduction). Fig. 4*D* shows that recombinant CHK1 was poorly phosphorylated on Ser³⁴⁵ in unstressed cells, but phosphorylation increased during the replication checkpoint. Surprisingly, N Δ 269 was strongly phosphorylated on Ser³⁴⁵, suggesting that the presence of the kinase domain may constrain the phosphorylation of Ser³⁴⁵ under unstressed conditions. Collectively, our results indicate that despite the lack of strong physical association between the kinase domain and the regulatory domain when they are separately expressed, they appear to mutually affect each other.

CHK1 but Not CHK2 Is Phosphorylated during Mitosis—It is well established during DNA damage or replication block that CHK1 is phosphorylated at several sites including Ser³⁴⁵ (see Introduction). Interestingly, we found that CHK1 was also phosphorylated during mitotic block. Fig. 5*A* shows that endog-

enous CHK1 was unphosphorylated in unstressed cells. As expected, CHK1 was phosphorylated during replication block induced by HU or aphidicolin (interestingly, thymidine did not efficiently induce CHK1 phosphorylation). The S phase block was verified by the mobility shifts of CDC2 (representing Thr¹⁴/Tyr¹⁵ phosphorylation) and the accumulation of cyclin A, cyclin B1, and cyclin E. The spindle dissociation poison NOC activates the spindle assembly checkpoint and locks cells in a mitotic state (55). Significantly, NOC also induced mobility shifts of CHK1 (lane 5). The dephosphorylation of CDC2, the accumulation of cyclin B1, and the destruction of cyclin A and cyclin E confirmed the efficacy of the mitotic block. Although NOC-induced mobility shifts of CHK1 were readily observed, the precise pattern of the bands depended on the particular running conditions and the resolving power of the SDS-PAGE. Highly complex patterns were occasionally observed (Fig. 5*B*), indicating that multiple sites were phosphorylated during mitotic block.

To delineate the temporal relationship between CHK1 phosphorylation and mitotic block, cell extracts were prepared at different time points after NOC treatment (Fig. 5*C*). As expected, histone H3 phosphorylation (an event that usually begins before prophase (56)) increased progressively following NOC treatment. Despite the constant level of total CDC2, there

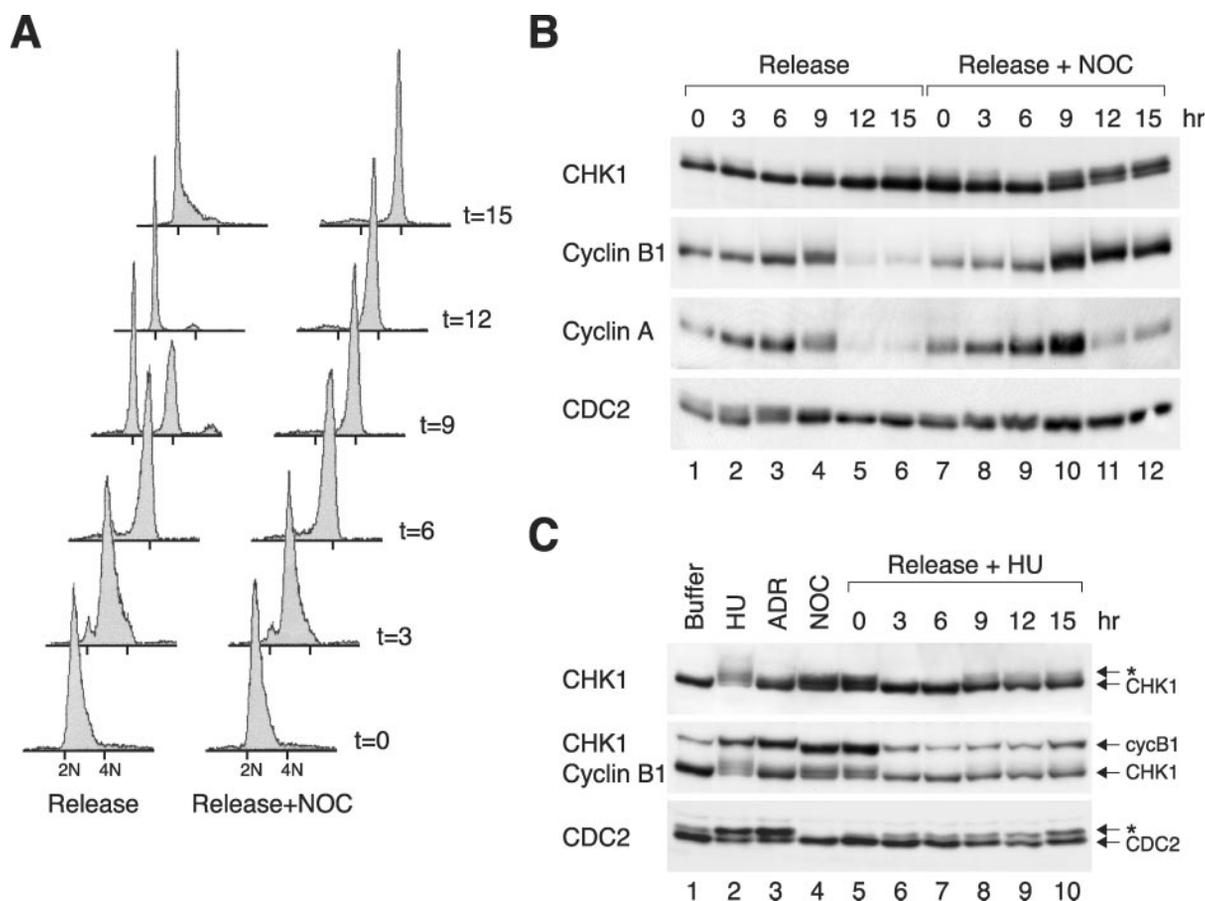


FIG. 6. CHK1 is dephosphorylated after release from mitosis. *A*, double thymidine synchronization: cell cycle analysis. HtTA1 cells were synchronously released into the cell cycle from a double thymidine block. Cells were either untreated (*lanes 1–6*) or treated with NOC (*lanes 7–12*) at 3 h after the release. Cells were harvested at the indicated time points and subjected to flow cytometry analysis. The positions of 2N and 4N DNA content are indicated. *B*, double thymidine synchronization: immunoblotting. Cells were synchronized as described for *A*. Cell extracts were prepared from cells harvested at the indicated time points. CHK1, cyclin B1, cyclin A, and CDC2 were detected by immunoblotting. *C*, mitotic release synchronization. HtTA1 cells were synchronously released into the cell cycle from a NOC block (*lanes 5–10*). HU was added at 3 h after release to trap cells in S phase. Cell extracts were prepared from cells harvested at the indicated time points. CHK1, cyclin B1, and CDC2 were detected by immunoblotting. Cells treated with buffer, HU, adriamycin (ADR), or NOC for 16 h were loaded as controls. The asterisks indicate the positions of the phosphorylated forms of CHK1 and CDC2.

was a reciprocal decrease in the inhibitory phosphorylation of CDC2 (Tyr¹⁵). Phosphorylation of CHK1 increased as more cells were blocked in mitosis, reaching a maximum at 18 h.

To exclude the possibility that the mitotic phosphorylation of CHK1 was specific to a particular cell type, we demonstrated that CHK1 was also phosphorylated in NOC-treated Ramos cells (Fig. 5*D*), HL-60 (Fig. 5*E*), and lymphoblastoid cells (Fig. 5*F*). To exclude the possibility that the CHK1 phosphorylation was due to apoptosis sometimes associated with prolonged NOC treatment, apoptosis was induced in Ramos and HL-60 cells with tumor necrosis factor α , Fas antibodies, or TRAIL. Unlike checkpoint activation, apoptosis did not trigger mobility shifts of CHK1 (Fig. 5, *D* and *E*).

We next examined the phosphorylation of CHK1 in synchronized cell cycle. Cells were synchronized at early S phase with a double thymidine block procedure. Flow cytometry analysis shows that the cells progressed synchronously through S and G₂, reaching mitosis at ~9 h (Fig. 6*A*). NOC was added to one set of the samples to trap cells in the subsequent mitosis. This is verified by the accumulation of cells with 4N DNA content. In these synchronized cells, degradation of cyclin A commenced at 9 h after release, and degradation of both cyclin A and cyclin B1 was completed by 12 h, further confirming that the mitosis occurred reasonably synchronously at ~9 h (Fig. 6*B*). As expected, cyclin A was degraded, and cyclin B1 accumulated when NOC was added to trap cells in mitosis. We found that

CHK1 was not phosphorylated during much of the cell cycle. Moreover, significant CHK1 phosphorylation was not observed during normal mitosis (*lane 4*). In contrast, CHK1 phosphorylation was readily seen when the cells were not allowed to exit mitosis (*lanes 10–12*). It is likely that mitosis was too transient to be efficiently sampled by our synchronization procedures. It is notable that even in the presence of NOC, CHK1 was not completely phosphorylated.

To see whether the mitotic phosphorylation of CHK1 is reversible once the cell exits mitosis, NOC-blocked cells were washed extensively to release them into G₁ (Fig. 6*C*). Further progression through the cell cycle was inhibited by trapping the cells in S phase with HU. As expected, the abundance of cyclin B1 was high during mitotic block and decreased as the cells were released. Notably, CHK1 was dephosphorylated when the cells were released into G₁ (compare *lanes 5* and *6*; clearer in a lighter exposure in the *middle panel*). This indicates that the phosphorylation of CHK1 during mitotic block is reversible.

To show that CHK1 phosphorylation was due to mitotic block and not specific to the NOC itself, another mitotic blocker was used instead. Fig. 7*A* shows that both NOC and taxol (a microtubule polymerization drug) triggered CHK1 phosphorylation (*lanes 8* and *9*), strongly suggesting that the phosphorylation was a result of the mitotic state. For a further control, no CHK1 phosphorylation was observed when lovastatin (57) was added to block cells in G₁ (*lane 10*). We further confirmed that cells

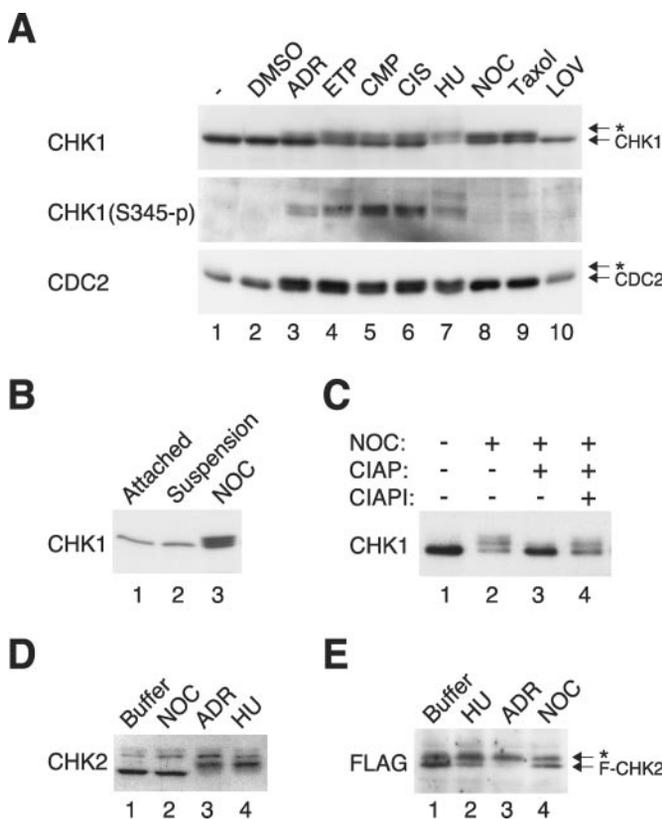


FIG. 7. Further characterization of mitotic phosphorylation of CHK1. *A*, mitotic phosphorylation of CHK1 is induced by both NOC and taxol and does not involve Ser³⁴⁵. HtTA1 cells were mock-treated or treated with Me₂SO (*DMSO*) carrier, adriamycin, etoposide, camptothecin, cisplatin, HU, NOC, taxol, or lovastatin as indicated. At 24 h after transfection, cell extracts were prepared and immunoblotted with antibodies against CHK1, Ser³⁴⁵-phosphorylated CHK1, or CDC2. The asterisks indicate the positions of the phosphorylated forms of CHK1 and CDC2. *B*, cell growth in suspension is not sufficient to induce CHK1 phosphorylation. HtTA1 cells were grown either under standard attached conditions or in suspension. Cell extracts were prepared, and CHK1 was detected by immunoblotting. Extracts of NOC-blocked cells were loaded in lane 3 as control. *C*, mobility shifts of CHK1 can be reversed by phosphatase treatment. HtTA1 cells were treated with either buffer or NOC for 16 h as indicated. Cell extracts were prepared, and CHK1 was immunoprecipitated. The immunoprecipitates were either untreated or treated with calf intestine alkaline phosphatase in the presence or absence of phosphatase inhibitors. CHK1 was detected by immunoblotting. *D*, endogenous CHK2 is phosphorylated after DNA damage and replication block but is not phosphorylated during mitotic block. HtTA1 cells were treated with buffer, NOC, adriamycin, or HU for 16 h as indicated. Cell extracts were prepared, and CHK2 was detected by immunoblotting. *E*, phosphorylation of recombinant CHK2 does not increase during mitotic block. HtTA1 cells were transfected with plasmids expressing FLAG-CHK2. At 24 h after transfection, the cells were treated with the indicated agents for another 16 h. Cell extracts were prepared, and FLAG-tagged proteins were detected by immunoblotting.

grown under both standard conditions and in suspension did not contain phosphorylated CHK1 (Fig. 7*B*), indicating that the phosphorylation of CHK1 was not simply due to the difference between attached and rounded up, detached cells (typical of cells in mitosis). To formally show that the mitosis-induced mobility shifts of CHK1 was due to phosphorylation, phosphatase treatment of the CHK1 immunoprecipitates was able to reverse the mobility shifts (Fig. 7*C*).

We next investigated whether CHK2 was also phosphorylated during mitotic block. We found that endogenous CHK2 was not phosphorylated when treated with NOC but was phosphorylated after DNA damage (adriamycin) or replication block (HU) (Fig. 7*D*). As shown above, ectopically expressed CHK2 was partially phosphorylated (see Figs. 2*E* and 4*A*). Fig. 7*E*

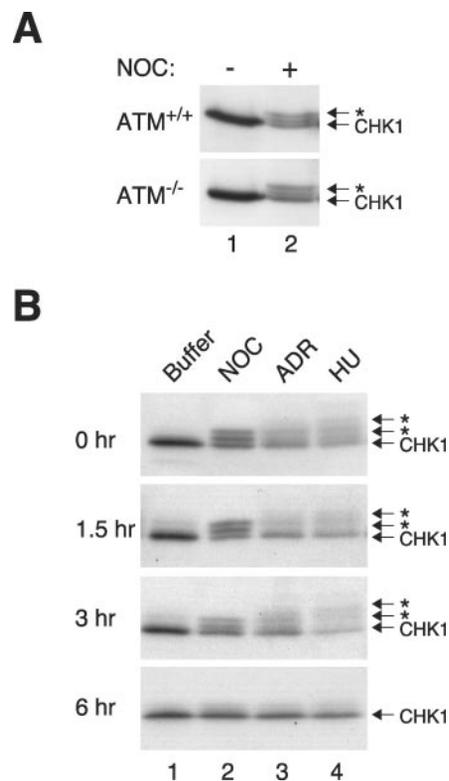


FIG. 8. Phosphorylation of CHK1 does not require ATM but is caffeine-sensitive. *A*, ATM-independent phosphorylation of CHK1 during mitotic block. Wild type or ATM^{-/-} lymphoblastoid cells were treated with either buffer or NOC for 16 h. Cell extracts were prepared, and CHK1 was detected by immunoblotting. The asterisk indicates the position of the phosphorylated forms of CHK1. *B*, mitotic phosphorylation of CHK1 is abolished by caffeine. HtTA1 cells were treated with the indicated drugs for 16 h. Caffeine was added, and the cells were harvested at the indicated time points. Cell extracts were prepared, and CHK1 was detected by immunoblotting. The asterisks indicate the positions of the phosphorylated forms of CHK1.

shows that adriamycin and HU stimulated further phosphorylation of FLAG-CHK2. However, no significant increase in phosphorylation was seen when cells were treated with NOC. Taken together, these data indicate that CHK1, but not CHK2, is phosphorylated during mitotic block.

Mitotic Phosphorylation of CHK1 Does Not Involve Ser³⁴⁵ and Is Independent on ATM—CHK1 is phosphorylated on Ser³⁴⁵ following treatments with HU or a variety of DNA-damaging agents, including adriamycin and etoposide (topoisomerase II poisons), camptothecin (topoisomerase I poison), and cisplatin (Fig. 7*A*). CHK1 in control cells or cells treated with the Me₂SO carrier alone did not display Ser³⁴⁵ phosphorylation. Interestingly, although both NOC and taxol induced phosphorylation of CHK1, Ser³⁴⁵ phosphorylation was at background level after treatment with these reagents (lanes 8 and 9). This experiment indicates that the mitotic phosphorylation of CHK1 occurs at sites other than the major ATM/ATR phosphorylation site.

ATM and ATR are required for the activation of CHK1 in response to DNA damage or replication block. We next investigated whether the mitotic phosphorylation of CHK1 also requires ATM. Lymphoblastoid cells from age-matched normal and AT individuals were treated with NOC. Fig. 8*A* shows that NOC-induced CHK1 phosphorylation occurred in both wild type and ATM^{-/-} lymphoblastoid cells, indicating that ATM is not required for the phosphorylation of CHK1 during mitotic block. Caffeine can bypass DNA damage and replication checkpoints by inhibition of ATM and ATR. As expected, caffeine treatment abolished the adriamycin- or HU-induced CHK1

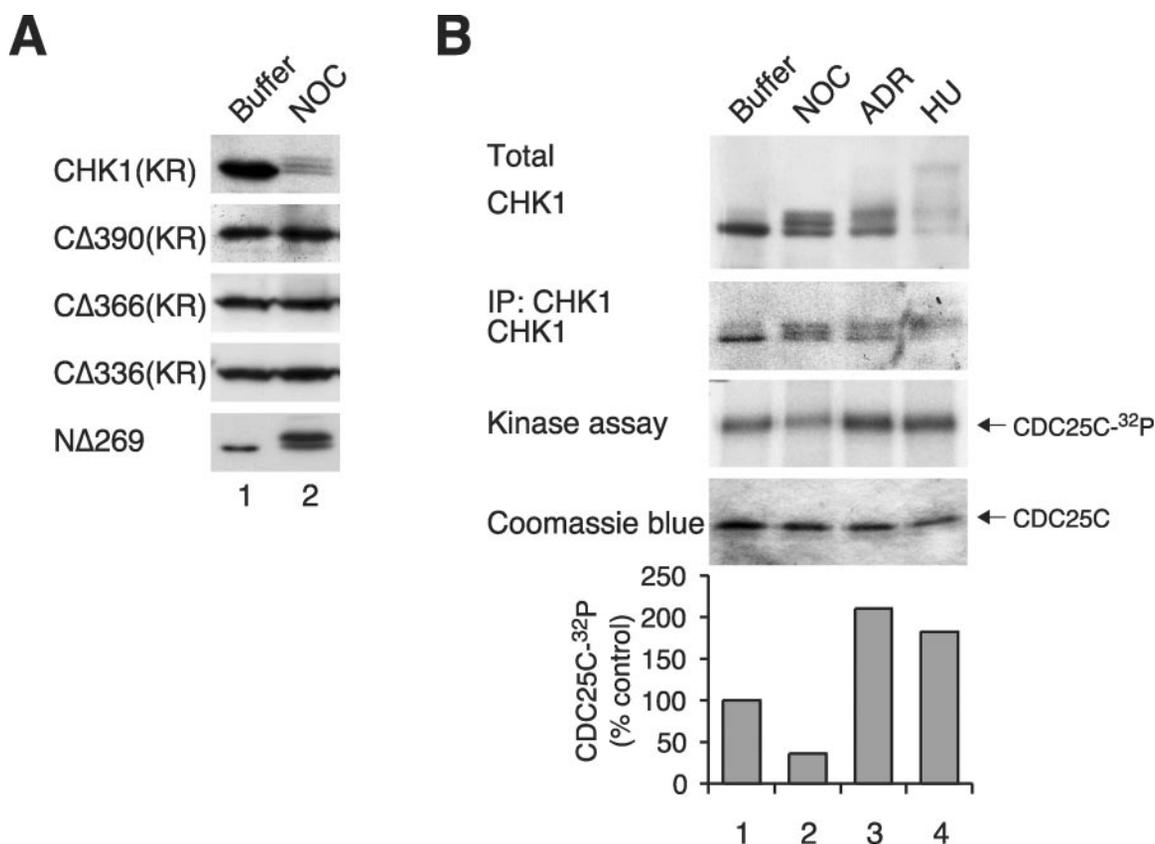


FIG. 9. Mitotic phosphorylation decreases CHK1 activity. *A*, the regulatory domain of CHK1 is necessary and sufficient for mitotic phosphorylation. Plasmids expressing FLAG-tagged and kinase-inactive (K38R) CHK1, C Δ 390, C Δ 366, and C Δ 336 as well as HA-CHK1(N Δ 269) were transfected into HtTA1 cells. At 24 h after transfection, the cells were treated with NOC for another 16 h before they were harvested for cell extract preparation. FLAG- and HA-tagged proteins were detected by immunoblotting. *B*, the activity of CHK1 is reduced during mitotic block. HtTA1 cells were treated with buffer or the indicated drugs for 16 h. Cell extracts were prepared, and CHK1 was detected by immunoblotting (*top panel*). The cell extracts were subjected to immunoprecipitation (*IP*) with CHK1 antiserum, and the kinase activity against GST-CDC25C_{195–259} was assayed. Phosphorylation was detected and quantified with a PhosphorImager. Uniform loading of GST-CDC25C_{195–259} was confirmed by Coomassie Blue staining. The presence of CHK1 in the immunoprecipitates was confirmed by immunoblotting after the kinase assay (*bottom panel*).

phosphorylation (Fig. 8B). Interestingly, the NOC-induced CHK1 phosphorylation was similarly inhibited by caffeine. Collectively, these data indicate that mitotic phosphorylation of CHK1 does not occur at Ser³⁴⁵, independent of ATM, but is dependent on other caffeine-sensitive pathways.

Mitotic Phosphorylation of CHK1 Depends on the Regulatory Domain and May Inhibit CHK1—To have an idea on the phosphorylation sites, CHK1 truncation mutants were expressed in mammalian cells before NOC treatment. As described above, several truncation mutants of CHK1 were autophosphorylated and may obscure the NOC-induced phosphorylation. To exclude the possibility of autophosphorylation, we introduced kinase-inactive mutations (K38R) into the truncation mutants. Fig. 9A shows that full-length CHK1(K38R) was phosphorylated after NOC treatment. In contrast, none of the C-terminally truncated mutants displayed mobility shifts after NOC treatment. Importantly, the regulatory domain alone was phosphorylated after NOC treatment, indicating that it is sufficient to account for at least part of the mitotic phosphorylation. These results suggest that mitotic phosphorylation of CHK1 was due to the action of other kinases and not to autophosphorylation and that the C-terminal regulatory domain may contain the phosphorylation sites.

To elucidate the potential effects of NOC-induced CHK1 phosphorylation on its activity, the kinase activity of endogenous CHK1 against GST-CDC25C_{195–259} was assayed (Fig. 9B). As expected, the kinase activity of CHK1 increased about 2-fold after treatment with adriamycin or HU. We found that the

kinase activity of CHK1 was consistently reduced after NOC treatment. In contrast, we have no evidence that CHK2 is inhibited during mitotic block.²

DISCUSSION

The Regulatory Domains of CHK1 and CHK2 Have Opposite Effects on the Basal Enzymatic Activity—Until recently, ATM-CHK2 and ATR-CHK1 have been thought of as largely linear, nonoverlapping pathways (14, 18, 25). More recent experiments suggest that these pathways are not mutually exclusive (26, 58). The fact that both CHK1 and CHK2 can be activated by ATM and ATR and can phosphorylate common targets (including CDC25A and CDC25C) suggests that CHK1 and CHK2 could be regulated by similar mechanisms. It is hence surprising that the two kinases are controlled differently and that the regulatory domains play very different roles.

We found that ectopically expressed CHK1 displayed relatively low kinase activity against CDC25C *in vitro* (Fig. 2C). Autophosphorylation, as assayed by gel mobility shifts (Fig. 2, A and B) and ³²P labeling (Fig. 2C), was also only slightly above background. In contrast, ectopically expressed CHK2 displayed mobility shifts, autophosphorylation, and activity against CDC25C (Fig. 2E). Disrupting the regulatory domains of CHK1 and CHK2 also has contrasting effects on the kinase activities. Whereas deletion of part of the CHK1 regulatory domain activated the kinase (Fig. 2, B and C), deletion of the CHK2 regulatory domain inactivated the kinase (Fig. 2, E and G). The ability of CHK1 and CHK2 mutants to hinder cell cycle progression

correlated with their *in vitro* kinase activities (Figs. 1 and 3). We propose that the protein kinase domain of CHK1 contains intrinsic activity but is suppressed by the regulatory domain. Conversely, the protein kinase domain of CHK2 does not contain intrinsic activity but is activated by the regulatory domain.

Interestingly, whereas removal of the C-terminal region activated CHK1, further deletion of the regulatory domain (like C Δ 309 and C Δ 284) decreased both autophosphorylation and CDC25C kinase activity (Fig. 1). One possibility is that these truncations may delete the autophosphorylation sites; this may not be the entire explanation, because removal of the autophosphorylation sites should not inactivate the kinase activity against CDC25C. Another possibility is that a potential kinase-activating region is eliminated in C Δ 309. It is also possible that the two events (autophosphorylation and activity toward external substrates) are linked, with the complete activation of the kinase may depend on autophosphorylation. C Δ 366, C Δ 352, C Δ 336, and C Δ 309 progressively removed one of the four SQ sites in the region. Phosphorylation of the remaining SQ sites may be responsible for the activation of C-terminally truncated CHK1. However, we found that there was no significant increase in Ser³⁴⁵ phosphorylation in the activating mutants.² Intriguingly, Ser³⁴⁵ was strongly phosphorylated when the kinase domain was removed (N Δ 269) (Fig. 4D). This suggests that the kinase for Ser³⁴⁵ is already present in unstressed cells and that the folding of CHK1 may restrain Ser³⁴⁵ phosphorylation. It is conceivable that the initial activation event of CHK1 is not Ser³⁴⁵ phosphorylation, but binding to other proteins or phosphorylation of other sites may open up the regulatory domain for Ser³⁴⁵ phosphorylation.

One model is that whereas the SQ/TQ domain stimulates the kinase domain, this is overcome by the suppression from the C terminus. The C terminus could act directly on the kinase domain or could inhibit the activating potential of the SQ/TQ domain. Stress-induced phosphorylation of the SQ/TQ domain may remove the constraints of the C terminus on the kinase domain. The difficulty in testing this model is that no direct interaction was detected between the kinase domain and the regulatory domain of CHK1 (Fig. 4C). It is possible that the putative interactions are weak and transient, which may well be sufficient to have a profound effect on the enzyme when the two domains are linked together. Another possibility is that the regulatory domain affects the kinase domain simply through steric hindrance, blocking the access of the kinase to Mg²⁺-ATP or substrates.

The fact that ectopic expressed CHK1 was not active as a kinase and did not arrest the cell cycle may explain why actively proliferating hepatocellular carcinoma can express a high level of CHK1.² One implication of this is the possibility of exploiting the stockpile of dormant CHK1 for treatment of cancers like hepatocellular carcinoma.

CHK2 can form oligomers through the FHA domain of one molecule and the SQ/TQ domain of another (see Introduction and Fig. 4A). Only very weak binding between the regulatory domain and the kinase domain could be detected (Fig. 4B). Unlike CHK2, we found no evidence of oligomers with the various CHK1 constructs (Fig. 4B and data not shown). Since autophosphorylation was required for CHK2 to form oligomers (Fig. 4A), it is not surprising that truncation mutants of CHK2 (which do not autophosphorylate) did not oligomerize. One important question is why the ectopically expressed CHK2 was phosphorylated but the endogenous CHK2 was not (Fig. 7, D and E). This may be explicable by a concentration-dependent effect, because activation of CHK2 may require oligomerization involving both inter- and intramolecular phosphorylation. Oligomerization could be promoted when CHK2 was overex-

pressed in mammalian cells or purified from *Escherichia coli*. We were not able to make a comparison with bacterially expressed CHK1 because of the insolubility of GST-CHK1 obtained after many attempts.²

The Regulatory Domain of CHK1 but Not That of CHK2 Is Involved in Phosphorylation during Mitotic Block—The delay in cell cycle during both the DNA damage checkpoint and replication checkpoint is carried out through the sequential action of ATM/ATR-CHK1/CHK2-CDC25A/CDC25C and eventually results in the inhibitory phosphorylation of CDC2/CDK2. The spindle assembly checkpoint, on the other hand, maintains a high level of cyclin B1-CDC2 activity through the stabilization and activation of CDC25A and CDC25C. One possibility is that the decrease in CHK1 activity may contribute to the activation of CDC25A/CDC25C. We found that CHK1 activity is low (but above background) in unperturbed cell cycle and indeed decreased during the spindle assembly checkpoint (Fig. 9B). The inactivation of CHK1 during mitotic block correlated with the phosphorylation of CHK1 (Fig. 5). Phosphorylation of CHK1 was triggered by both NOC- and taxol-induced spindle assembly checkpoint (Fig. 7A), occurred in a variety of cell types (Fig. 5), and was reversed by phosphatase treatment (Fig. 7C). Moreover, this phosphorylation did not involve Ser³⁴⁵ (Fig. 7A) or ATM (Fig. 8A) but was sensitive to caffeine (Fig. 8B), suggesting the possibility of ATR participation.

Other than excluding Ser³⁴⁵, the mitosis-specific phosphorylation sites in CHK1 are not pinpointed here. What is clear is that phosphorylation does not require autophosphorylation and disruption of the C-terminal region of the regulatory domain inhibited phosphorylation (Fig. 9A). Moreover, the regulatory domain alone is sufficient for at least some of the mitosis-specific phosphorylation events (Fig. 9A), although this is complicated by the fact that new phosphorylation events may occur when the regulatory domain is expressed alone (like Ser³⁴⁵ phosphorylation) (Fig. 4D). Phosphorylation is likely to occur on multiple sites, because a complex pattern of bands was observed on SDS-PAGE (Fig. 5B). Without identification of the precise phosphorylation sites, it is difficult to formally demonstrate that mitotic phosphorylation inhibits CHK1. One obvious kinase that is activated during mitosis is CDC2. Other mitosis-specific kinases include the polo-like kinases and the aurora kinases. If cyclin B1-CDC2 complexes do indeed phosphorylate CHK1, this would elegantly complete a positive feedback loop comprising CHK1-CDC25A/CDC25C-CDC2. It is notable that CHK1 contains four potential CDC2 phosphorylation sites (three SP and one TP), all within the regulatory domain.

In conclusion, the regulatory domains of CHK1 and CHK2 modulate the activity of the kinase domain in very different manners. This includes the effects on the basal activity of the kinases, the ability to form dimers, and the phosphorylation during mitotic block.

Acknowledgments—We thank Drs. Benjamin Bonavida, Tony Hunter, Wai Jiang, Helen Piwnicka-Worms, and Katsumi Yamashita for generous gifts of reagents. Many thanks are due to members of the Poon laboratory for constructive criticism of the manuscript.

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