Regulation of Chk1 Includes Chromatin Association and 14-3-3 Binding following Phosphorylation on Ser-345*  

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Checkpoints are biochemical pathways that provide the cell with mechanisms to detect DNA damage and respond by arresting the cell cycle to allow DNA repair. The conserved checkpoint kinase Chk1 regulates mitotic progression in response to DNA damage and replication interference by blocking the activation of Cdk1/cyclin B. Chk1 is phosphorylated on Ser-317 and Ser-345 following a checkpoint signal, a process that is regulated by Atr, and by the sensor complexes containing Rad17 and Hus1. We show that Chk1 is associated with chromatin in cycling cells and that the chromatin-associated Chk1 is phosphorylated in the absence of exogenous DNA damage. The UV-induced Ser-345-phosphorylated forms of Chk1 that appear minutes after treatment are predominantly associated with chromatin. The Ser-345 site is in a 14-3-3 consensus binding motif and is required for nuclear retention of Chk1 following an hydroxyurea-induced checkpoint signal; nonetheless, Ser-345 or Ser-317 are not required for the chromatin association of Chk1. Hus1, a member of the proliferating cell nuclear antigen-like damage recognition complex plays a role in the phosphorylation of Chk1 on Ser-345, however, Hus1 is not required for phosphorylation on Ser-317 or for Chk1 localization to chromatin. These results indicate that there is more than one step in Chk1 activation and that the regulation of this checkpoint signaling is achieved at least in part through phosphorylation of Ser-345, which serves to localize Chk1 in the nucleus presumably by blocking Crm1-dependent nuclear export.

The cellular response to DNA damage and replication interference involves the activation of signal transduction pathways known as checkpoints that prevent cell cycle progression and induce the transcription of genes that facilitate DNA repair (1). These responses ensure that DNA replication and chromosomal segregation are completed with high fidelity. Defects in checkpoint responses in higher organisms can result in genomic instability, cell death, and a predisposition to cancer (2).

Mammalian checkpoint genes include the tumor suppressor genes P53 and ATM. The Atm protein is required for several aspects of the response to DNA damage including cell cycle arrest and p53 activation following ionizing radiation (3, 4). Atr, and the DNA-activated kinase (DNA-PKCs), are homologs of the Atm family of protein kinases. This family includes the checkpoint proteins Mec1 and Tel1 from Saccharomyces cerevisiae and rad3 from Schizosaccharomyces pombe.

Checkpoint kinase 1 (Chk1) was first identified in S. pombe because of its role in the checkpoint arrest at G2/M (5, 6). Chk1 proteins function downstream of the Atm-like proteins in the yeasts and higher eukaryotes to regulate the checkpoint response that prevents chromosome segregation in the presence of DNA damage. In mammalian cells and S. pombe, Chk1 blocks mitotic progression via inactivation of the mitotic inducer Cdc25 (7–9). Another conserved kinase that functions downstream of the Atm-like kinases and regulates Cdc25 is Chk2/Cds1. An additional role for Chk2 (hCds1) and Chk1 in mammals is to phosphorylate p53 on Ser-20, Ser-15, and Ser-37 (10–12).

The picture of how sensor complexes activate the checkpoint kinases following DNA damage has begun to emerge. Because of their association with DNA and predicted protein structure, at least three complexes have been assigned the role of sensors in yeast and mammals. The Rad17 “clamp loading” complex (13), which is associated with replication factor C-like proteins, and the Rad9-Hus1-Rad1 “clamp-like” complex (14–17), which has been proposed to act like a proliferating cell nuclear antigen clamp are two such complexes. A third complex that contains Atr/Atrip (18) requires both the Rad17 and Hus1 complex for activation of the DNA damage response (Ref. 19, and references therein). Following recognition or processing of the DNA lesion, the Rad17 complex recruits the Rad9-Hus1-Rad1 complex to DNA in an analogous mechanism to replication factor C-mediated loading of proliferating cell nuclear antigen onto DNA during DNA replication. Then, the Rad17 protein becomes phosphorylated by Atr (20) in a Hus1-dependent manner (19). Rad17 phosphorylation in response to high doses of ionizing radiation is reduced in Atm (−/−) cells, which suggests that Atm complexes interact with Rad17 in a similar manner to Atr complexes in response to ionizing radiation. Recently it has been shown that Atr localizes to chromatin independently of the Rad17 complex; however, the phosphorylation of Atr substrates and thus checkpoint signaling is regulated by the sensor complexes containing Rad17 and Hus1 (18, 19). These findings led to the model that Rad17 (and Hus1) complexes provide substrate specificity to the central checkpoint kinases Atr and Atm (19). All of these stepwise interactions argue that the
activation of the response could be lesion, cell cycle, and protein complex specific. Chk1, like Atr, is an essential kinase (21, 22) in mammals, and its activation via phosphorylation is dependent on Atr in Xenopus and mammals (21, 23, 24). Chk1 has been shown to be phosphorylated on Ser-345 following DNA damage (21, 25). Phosphorylation on Ser-345 is blocked in cells lacking the Atr kinase (24). The kinase is reconstituted in cells lacking the Hss1 component of the putative sensor complex formed with Rad1 and Rad9 (26) and in cells with reduced levels of the Rad17 protein (19). Thus, several complexes associated with DNA including the Atr-Atrip complex regulate the phosphorylation of Chk1.

Here we show that Chk1 also localizes to chromatin and that phosphorylation of Ser-345 and Ser-317 is likely to take place on the chromatin-associated Chk1. Furthermore, we show that phosphorylation of Ser-345 serves to localize Chk1 in the nucleus presumably by blocking Crm1-dependent nuclear export.

EXPERIMENTAL PROCEDURES

Cloning, Mutagenesis, DNA, and Protein Purification—The GFP-Chk1 and GFP-Chk1D132 constructs have been described elsewhere. Site-directed mutagenesis was performed using the QuikChange kit by following the manufacturer’s instructions (Stratagene). GST fusion proteins were expressed in *Escherichia coli* (BL21). Soluble proteins were concentrated using glutathione-Sepharose beads or the proteins were expressed in Hi5 insect cells and collected as previously described (7).

Cell Culture, Treatment of Cells, and Transfection—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Mouse embryo fibroblasts were cultured on gelatin-coated plates in Dulbecco’s modified Eagle’s medium + 15% fetal calf serum. Cells were treated with 50 μg/ml methimethane sulfonate (MMS) for 2–4 h, 5 mM hydroxyurea (HU) for 18 h, or 2 μg/ml Leptomycin B for 2 h. HeLa cells were transfected using FuGENE 6 (Roche Diagnostics) transfection reagent and 293T cells were transfected with LipofectAMINE (Invitrogen) following the manufacturer’s instructions. The delivery of morpholinos was carried out essentially according to manufacturer’s instructions for special delivery protocol (Gene Tools, LLC). Briefly, the caspase or invert control morpholinos were added to 3 million cells 5 h after seeding in 75-cm² flasks. Three hours later the cells were plated onto flasks and plates for (UV irradiation). Twenty-four hours after delivery, HU was added to 1 set and 15 h later another set was irradiated with 50 J/m² UV and harvested 2 h later along with control and HU treated sets. Sequence for caspase-AS was CCTCA-GAACCACCTGCGCTGCTCAT and invert caspase control oligo sequence was TACTGTCGCCCTCACCAAGACTCC.

Cell Fractionation Experiments—For experiments with microccocal nuclease (Fig. 1A): HeLa cells were collected and washed with phosphate-buffered saline and fractionated as described with minor modifications (27). Cells were re-suspended in 200 μl of solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 mM sucrose, 10% glycerol, 1 mM dithiothreitol, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors) and incubated for 10 min on ice. Cytoplasmic proteins (C) were separated from nuclei by low-speed centrifugation (17400 g for 4 min at 4 °C). Isolated nuclei were re-suspended in 100 μl of solution A containing 1 mM CaCl₂ (Triton X-100 was added to a final concentration of 0.1% wherever mentioned), and the nuclei were left on ice for 5 min. The nuclei were centrifuged as above and the supernatant (N1) was removed. Nuclei were re-suspended in 100 μl of solution A with 1 mM CaCl₂ and containing or not 50 units of microccocal nuclease (Amersham Biosciences), and incubated at 37 °C for 10 min before the addition of EGTA and EDTA to 1 mM as final concentration to stop the reaction. Nuclei were pelleted down by the same gentle centrifugation and the supernatant was combined with N1 above. Nuclei were re-suspended in 200 μl of solution B (3 mM EDTA, 0.2 mM EGTA, and 1 mM dithiothreitol). After 30 min incubation on ice, supernatant (N2) were separated from chromatin (Ch) by centrifugation (17000 × g for 4 min at 4 °C). Isolated chromatin was washed once with solution B and centrifuged at high speed (10,000 × g for 1 min at 4 °C). Finally, chromatin was re-suspended in 200 μl of 2× loading buffer and sheared by sonication (9). To compare Chk1 localization on chromatin to that published previously for Atr, fractionation protocols were followed as described (19) (Figs. 3, 4 and E). HeLa cells were either transfected not and either treated or not with 5 mM HU for 18 h were collected and washed with phosphate-buffered saline and re-suspended in 200 μl of solution A. Triton X-100 was added to a final concentration of 0.1%, and the cells were left on ice for 5 min. Cytoplasmic proteins (C) were separated from nuclei (N) by low speed centrifugation (13000 × g for 4 min at 4 °C). An aliquot of the nuclei was saved (N2). The nuclei were re-suspended in 100 μl of solution A and incubated on ice for 10 min, and pelleted as above. The nuclei were then lysed in 100 μl of solution B. After 30 min incubation on ice, the supernatant (N) was separated from chromatin (Ch) by centrifugation (17000 × g for 4 min at 4 °C). Isolated chromatin was washed and sonicated as above. The supernatants from the second round of solution A were combined with N2 above. To determine the SDS-PAGE step (N). All other experiments (Figs. 1B, 3, B, D, 4A and B) were carried out using this fractionation protocol except that Triton X-100 was omitted.

Pull-down Assays—Extracts prepared from Hi5 insect cells infected with hChk1Δ, hChk1-Δ132, hChk1S345A expressing baculovirus were incubated with bacterially expressed GST, and GST-14-3-3 bound to glutathione beads in 10 mM Hepes (pH 7.5), 50 mM glycero-phosphate, 200 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 0.5% Nonidet P-40, 5 μM ATP, 1 mM dithiothreitol with protease and phosphatase inhibitors for 2 h at 25 °C. The beads were gently precipitated, washed in the same buffer 3 times, resuspended in SDS sample buffer, boiled, and subjected to SDS-PAGE. After transfer to polyvinylidene difluoride membrane, the Chk1 proteins were detected with anti-Chk1 antibodies that recognize the NH₂ terminus of Chk1 (G4, Santa Cruz Biotechnology).

Chk1 Binding Assays—Eight hundred μg of whole cell extracts prepared from HeLa cells treated with 5 mM HU for 20–24 h were incubated with an equal amount of bacterially expressed GST, GST- hChk1(1WT), or GST-hChk1S345A bound to glutathione beads in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2 mM MgCl₂, 5 mM MnCl₂, 2 mM dithiothreitol, 10% glycerol, 250 mg of Ran-GTP, 2 mM ATP, 20 μM ATP with protease and phosphatase inhibitors for 2 h at 25 °C. The beads were gently precipitated and washed 4 times in 50 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.5% Nonidet P-40 containing all protease inhibitors, and fractionated on SDS-PAGE. After transfer the Crm1 proteins were detected using anti-Crm1 antibodies (H-300, Santa Cruz Biotechnology).

Immunoprecipitation Assays—Immunoprecipitation of EYFP-Chk1 was carried out as previously described (7) using anti-GFP antibodies (Molecular Probes). Immunoprecipitation of FLAG-Chk1 from 293T cells was carried out by first enriching for chromatin-associated proteins. 293T cells transfected with either CMV-FLAG-Chk1 or pCDNA3.1 were treated or not with 5 mM HU for 18 h, collected, and washed with phosphate-buffered saline and resuspended in solution A (see "Cellular Fractionation Experiments"). Cytoplasmic proteins were separated from nuclei by low-speed centrifugation at 4 °C. Isolated nuclei were washed with solution A and resuspended in solution A containing 1 mM CaCl₂ and 150 units of microccocal nuclease (Sigma); incubated for 1 h at 4 °C before stopping by adding 1 mM EGTA as final. Sodium chloride was added to 300 mM final concentration and incubated for 10 min on ice. The nuclear preparation was then sonicated twice for 15 s and cleared by centrifugation at 16,850 × g for 15 min at 4 °C. The extracts were diluted in Buffer Z (25 mM Hepes-KOH, pH 7.9, 50 mM KCl, 10 mM MgCl₂, and 0.1% Nonidet P-40) with a final concentration of 150 mM NaCl, 1 mM EGTA, and 0.1% Triton X-100 (plus phosphatases and proteases inhibitors). The extracts were pre-cleared with Protein A-Sepharose followed by incubation with the indicated antibody (1 h at 4 °C). The precipitated protein was eluted by gentle centrifugation, washed extensively, and separated on a 4–12% gradient SDS-PAGE.

In Vitro Kinase Assays—Immunoprecipitations of Chk1, Chk1D132, or Chk1S345A were carried out with anti-GFP antibodies and kinase reactions were performed in kinase buffer (15 μl of [γ-32P]ATP, 20 μM ATP). Immunoprecipitates were separated by SDS-PAGE on 4–12% acrylamide gels.

Western Blotting, Data Quantification, and Analysis—Western blot analyses were performed as previously described (7). Murine and human Chk1 were detected with monoclonal antibody G4 (Santa Cruz Biotechnology). EYFP fusion proteins were detected with either anti-GFP antibodies (G4, Santa Cruz Biotechnology) or mouse Chk1 antibodies (G4, Santa Cruz Biotechnology). Atr (Ab1) and Orc2 (Ab2) were detected with antibodies from Oncogene Research; Crm1, 14-3-3, and p53 (DO1) were detected with antibodies from Santa Cruz Biotechnology. Caspase was detected with affinity purified rabbit polyclonal antibodies to a peptide.
Chk1 Localizes to Chromatin in Cycling Cells—We have observed that Chk1 protein binds DNA cellulose, double strand breaks, and single strand DNA. There are two likely explanations for how Chk1 would bind to DNA: either Chk1 may be part of a chromatin-associated sensor complex and becomes activated while on chromatin; or Chk1 may be recruited to chromatin upon activation to modulate the checkpoint response and/or DNA repair. The association of Atr, the kinase that acts upstream of Chk1, with chromatin does not depend on a checkpoint signal (19, 23) nor on Rad17 (19) indicating that there are two separate complexes that bind DNA in the absence of a checkpoint signal. To test whether Chk1 also binds chromatin in vivo, and whether this association was regulated by a checkpoint signal we carried out cell fractionation experiments. Chk1, like Atr, was associated with the chromatin fraction (Ch) in cycling cells and was removed by incubation of nuclei with micrococcal nuclease as evidenced by its appearance in the nuclear soluble fraction (N1) (Fig. 1A, compare N1 with and without MNase treatment). Concomitantly with an increase of Chk1 levels in the soluble nuclear fraction, we observed an increase in the levels of the chromatin-associated Orc2 in the soluble nuclear fraction following treatment with nuclei. The MNase treatment resulted in release of Chk1 to the N1 fraction whereas Orc2 required lysis of the nuclei to be released to the soluble nuclear fraction (N2). This could be because of the size or nature of the proteins or protein complexes. It is possible that Chk1 once released from chromatin could diffuse out of the nucleus, whereas Orc2 complexes could not. These data indicate that a fraction of Chk1 is associated with DNA in the absence of treatment with agents that cause DNA damage or replication blocks. The levels of chromatin-associated Chk1 did not increase dramatically following HU treatment, however, we did observe a slower migrating form of chromatin-associated Chk1, presumably because of phosphorylation (Fig. 1B), which was also detected in the longer exposures of the untreated samples, suggesting that phosphorylated Chk1 associates with chromatin and could possibly become phosphorylated while on chromatin.

Chk1 Localizes to the Nucleus during a HU-induced Replication Block and after MMS Treatment—Chk1 localizes to distinct nuclear structures during S phase (7, 28). To determine whether the localization of Chk1 is regulated following a checkpoint-induced response, we generated fusion proteins by attaching EYFP in-frame at the amino terminus of full-length Chk1 proteins, one that retains catalytic activity and another that is catalytically inactive. HeLa cells transfected with the EYFP vector alone or the EYFP fusion constructs were treated or not with HU or MMS, and the subcellular localization of the EYFP and EYFP fusion proteins was determined by fluorescence microscopy (Fig. 2). EYFP-Chk1 was observed both in the nucleus and cytoplasm of cells (Fig. 2, arrow) with a stronger signal in the nucleus of a small percentage of cells from an asynchronous population (Fig. 2, arrowhead). Following chronic treatment with 5 mM HU (19 h) or 50 μg/ml MMS (4 h), the Chk1 signal was localized in the nucleus (Fig. 2, graph). Because both HU and MMS synchronize cells in S phase, it is possible that the checkpoint signal-induced nuclear localization/retention of Chk1 could be because of a cell cycle-specific effect. The duration of the MMS treatment (4 h) was not sufficient to synchronize the entire population of cells in S phase, suggesting that the increase in nuclear accumulation of Chk1 was triggered in response to MMS-induced DNA damage. We also examined cells transfected with EYFP-Chk1Δ and EYFP-Chk1KD, which are deletion mutants missing the C-terminal 115 amino acids (361–476) that contain two conserved domains present in all Chk1 orthologues. The deletion mutation did not alter the checkpoint-induced localization/retention of Chk1, although an increase in the number of cells with abnormal nuclear and cellular morphologies was observed in the cells transfected with these constructs.

FIG. 1. Chk1 localizes to chromatin. A, cycling HeLa cells were fractionated to obtain a cytoplasmic fraction and nuclei. The nuclei were further treated or not with micrococcal nuclease (MNase) as described under “Experimental Procedures.” The proteins in each fraction were separated in parallel by SDS-PAGE and the duplicate membranes were blotted with antibodies against Chk1, Atr, and Orc2. C, cytoplasmic; N1, soluble nuclear fraction with or without micrococcal nuclease treatment; N2, soluble nuclear proteins after lysis of nuclei; Ch, pellet after lysis of nuclei. B, HeLa cells treated or not with HU were fractionated as in Ref. 19 without the addition of Triton X-100 at the initial incubation step. C, cytoplasmic; N, soluble nuclear proteins after brief incubation in MNase; Ch, chromatin fraction.

FIG. 2. Chk1 localization changes in response to DNA damage and replication blocks. Left, HeLa cells transfected with EYFP-Chk1 treated or not with HU or MMS as described under “Experimental Procedures.” The expressed proteins were visualized by fluorescence microscopy. Right, graph depicting the quantification of EYFP localization in HeLa cells transfected with the EYFP vector, EYFP-Chk1, or EYFP-Chk1KD. The graph shows the percentage of cells displaying signal almost exclusively in the nucleus (arrowhead left panel) from EYFP, EYFP-Chk1, or EYFP-Chk1KD, following treatment with (purple bars) or without (blue bars) HU. At least 100 cells were scored per condition. Cells that showed little difference between nuclear and cytoplasmic signal N = C were scored as not displaying nuclear localization (arrow left panel).
Chromatin Association of Chk1

These results indicate that the kinase activity and carboxyterminal domains contained in the last 115 amino acids of Chk1 are not required for the nuclear localization/retention of Chk1 induced by HU or DNA damage. We also observed the checkpoint-induced nuclear distribution of EYFP-Chk1 in fibroblasts, HT1080 (fibrosarcoma), and MCF7 (breast epithelial origin) cells. The checkpoint signal-induced localization of Chk1 is also not altered in cells lacking Atm (-/-), indicating that Atm is not required for this response, although these findings do not exclude a redundant role for Atm in this process. Chk1 is phosphorylated on Ser-317 and Ser-345 in response to DNA damage in an Atr-dependent manner (21, 24); we set out to investigate whether Chk1 nuclear retention and/or its association with chromatin was regulated by serine phosphorylation.

Chromatin-associated Chk1 Is Phosphorylated on Ser-317 and Ser-345—We observed that after HU treatment both the soluble nuclear and chromatin-associated Chk1 contained a slower migrating form, presumably because of phosphorylation (Figs. 1B and 3A). The current model of Chk1 activation predicts that Chk1 is phosphorylated on Ser-345 following Atr activation by the Rad17 and Rad1-Hus1-Rad9 complexes. We examined the Ser-345 phosphorylation status of the different pools of Chk1 by immunoblotting with phospho-specific antibodies. Both the chromatin-associated and soluble nuclear pools of Chk1 were phosphorylated on Ser-345 following treatment with HU or 50 J/m² UV radiation (Fig. 3A). We observed a weak phospho-Ser-345 signal in the chromatin-associated pool of Chk1 from untreated cells that correlated with the slower migrating form of Chk1 in chromatin from these cells (Figs. 1B and 3A). We also observed two forms of Chk1 (faster and slower migrating) that reacted with the phospho-Ser-345 antibody in the soluble nuclear fraction from the treated cells (Fig. 3A), suggesting that Ser-345 is one of at least two checkpoint-induced phosphorylation sites on Chk1. Recently, Chk1 has been shown to be phosphorylated on Ser-317, another Atr consensus site (24). Following treatment, the levels of Ser-317 phosphorylation were increased both in the soluble nuclear and chromatin pools of Chk1. We also observed phospho-Ser-317 and phospho-Ser-345 signals in the cytoplasmic fraction after treatment with HU and UV. This finding indicates that Chk1 is targeted to chromatin via Ser-317 and/or Ser-345 phosphorylation. To test this, we carried out fractionation experiments with cells expressing the S317A, S345A, and S317A/S345A mutated Chk1. For these assays we modified our fractionation experiments to compare these results with those published for Atr (19). When the detergent Triton X-100 was included in the hypotonic buffer, we observed an increase in the Chk1 signal present in the cytoplasmic fraction; however, the Chk1 signal (endogenous and EYFP-Chk1) in the chromatin fraction was not affected by this treatment. Chk1 remained associated with the detergent-insoluble chromatin fraction that also contained Atr (Fig. 3E). We observed that the Ser-317 or Ser-345 mutations did not affect the relative amounts of Chk1 associated with chromatin (Fig. 3E).

Chk1 Phosphorylated on Ser-345 following UV Radiation Is Detected First on Chromatin—One question that is raised by a model of checkpoint activation on chromatin and signaling is in regard to the mechanism by which the activated checkpoint kinases such as Chk1 and Chk2 phosphorylate non-chromatin-associated substrates such as Cdc25C in addition to chromatin-associated substrates like p53 (Fig. 3B). One possibility is that Chk1 becomes activated on chromatin and then dissociates to phosphorylate targets such as Cdc25. Alternatively, a soluble nuclear pool of Atr could phosphorylate a nuclear pool of Chk1 not associated with chromatin. To determine the relative kinetics by which the non-chromatin and chromatin-associated Chk1 pools are phosphorylated on Ser-345, and Ser-317 we followed the cellular distribution of total and phosphorylated Chk1 over time following UV radiation. Cycling HeLa cells were irradiated with 50 J/m² of UV and harvested at 15, 30, 60, 90, and 120 min following treatment. The cells were fractionated essentially as in Fig. 3A. As shown in Fig. 3C, the level of Ser-345-phosphorylated Chk1 in the chromatin fraction increased 15 min after UV treatment with a peak of phosphorylated protein at 120 min. In addition, the phospho-Ser-345 epitope was increased in the nuclear fraction 15–30 min after UV treatment and continued to increase during the course of the experiment. The phospho-Ser-317 epitope also increased in the soluble nuclear fraction at 15 min post-UV and the chromatin form of the phosphorylated protein became prominent 30 min after treatment. To rule out differences because of transfer of proteins to two membranes, and to better observe the changes in Chk1 protein during the entire course of the experiment we separated and transferred the proteins from all the soluble nuclear and chromatin fractions in a single gel (Fig. 3D). These data support our earlier findings that Chk1 is phosphorylated on Ser-345 in the absence of a radiation-induced checkpoint signal and that the DNA damage-induced phosphorylation of Chk1 on Ser-345 is detected first in the chromatin fraction before the accumulation of the soluble nuclear phosphoprotein, suggesting that phosphorylation on this residue takes place on chromatin-associated Chk1.

Phosphorylation at Ser-317 or Ser-345 Is Not Required for Chk1 Localization to Chromatin—Chk1 is associated with chromatin in untreated cells (Figs. 1 and 3), and we could detect phosphorylated Chk1 in the chromatin fraction of these cells. The possibility exists that Chk1 is targeted to chromatin via Ser-317 and/or Ser-345 phosphorylation. To test this, we carried out fractionation experiments with cells expressing the S317A, S345A, and S317A/S345A mutated Chk1. For these assays we modified our fractionation experiments to compare these results with those published for Atr (19). When the detergent Triton X-100 was included in the hypotonic buffer, we observed an increase in the Chk1 signal present in the cytoplasmic fraction; however, the Chk1 signal (endogenous and EYFP-Chk1) in the chromatin fraction was not affected by this treatment. Chk1 remained associated with the detergent-insoluble chromatin fraction that also contained Atr (Fig. 3E). We observed that the Ser-317 or Ser-345 mutations did not affect the relative amounts of Chk1 associated with chromatin (Fig. 3E).

The possibility exists that the association of Ser-317 or Ser-345 mutants with DNA is because of the interaction of the mutated proteins with chromatin indirectly via association with endogenous Chk1 protein. When both the exogenous and endogenous proteins are detected with the same antibody, there is considerably more EYFP-Chk1Ser-345 than endogenous protein associated with chromatin in these assays. Therefore, the amount of exogenous Chk1 bound to the chromatin argues against a Chk1-EYFP-Chk1 dimer mechanism.

These results indicate that Chk1 is regulated at several levels during the cell cycle and in response to a checkpoint signal. Chk1 is localized to chromatin in cycling cells where it is likely to be activated by the interaction of sensor complexes with Atr-Atrip. Phosphorylation of Ser-317 or Ser-345 is not required for the association of Chk1 with chromatin.

Hus1 Plays a Role in the Phosphorylation of Chk1 on Ser-345 but Is Not Required for Its Association with Chromatin—Phosphorylation of Chk1 on Ser-345 has been shown to be reduced in Hus1 (-/-) cells following treatment with HU, ionizing (IR), and ultraviolet radiation (UV) (26). Should the phosphorylation of Chk1 take place on DNA then the requirement of Hus1 could be to either localize Chk1 to chromatin, or to mediate the interaction of Chk1 complexes with Atr-Atrip complexes. We set out to determine whether Hus1 was required for Chk1 localization to chromatin. Hus1 (-/-), p21 (-/-), and Hus1 (+), p21 (-/-) (as control) mouse embryo fibroblasts were fraction-
FIG. 3. A, phosphorylated Chk1 associates with chromatin. HeLa cells were treated or not with HU (18 h treatment) or 50 J/m² UV and fractionated into cytoplasm (C) and nuclei (N) post-HU treatment and 2 h post-UV. The nuclei were further fractionated according to “Experimental Procedures” into soluble nuclear (N) and chromatin-associated insoluble fractions (Ch). The proteins were separated by SDS-PAGE and blotted with antibodies that recognize phospho-Chk1Ser-345, phospho-Chk1Ser-317, Chk1, Atr, and Orc2. The topmost bands in the phospho-Ser-345 panels are cross-reacting bands.

B, HeLa cells transfected with EYFP-CHK1 and treated or not with HU were fractionated with buffers containing 0.1% Triton X-100 as described under “Experimental Procedures.” The proteins were processed for Western analyses as in Fig. 1. N, proteins after lysis of nuclei; C, cytoplasmic proteins; Ch, chromatin. The proteins were detected with antibodies against Chk1, Atr, Cdc25C, p53, and Orc2.

C, HeLa cells were irradiated or not with 50 J/m² of UV and collected 15, 30, 60, 90, and 120 min following radiation. The levels of Chk1 and phosphorylation state, Atr and Orc2 levels were detected as above. N, proteins after lysis of nuclei; C, cytoplasmic proteins; Ch, chromatin. Ser-345 and Ser-317 phosphorylation are not required for Chk1 chromatin association.

D, the soluble nuclear protein and chromatin fractions from C were separated on the same SDS-PAGE gel and transferred to nitrocellulose. The levels of Chk1 and phosphorylation state, Atr and Orc2 levels were detected as above. N, soluble nuclear proteins after lysis of nuclei; Ch, chromatin fraction. E, EYFP-Chk1 and EYFP-Chk1S345A/S317A proteins associate with chromatin. HeLa cells transfected with EYFP-CHK1, EYFP-CHK1S345A, EYFP-CHK1S317A, or EYFP-CHK1S317A/S345A were fractionated with buffers containing 0.1% Triton X-100 as described under “Experimental Procedures.” The proteins were processed as above and detected using antibodies to Chk1, Atr, and Orc2.
phase. To test this, we examined the chromatin association and stress (29), which makes it a candidate protein to regulate plays a role in the activation of Chk1 during DNA replication also suggest that there are additional components that target can mediate Chk1 activation by Atr or possibly Atm. Our data processing. There is residual Chk1 phosphorylation in cells for Atr and Chk1 to interact at the site of DNA damage/lesion are determined by Western analyses. The association of Chk1 amounts of Chk1, phospho-Ser-317, and phospho-Ser-345 Chk1 protocols and UV as previously described (26) (Fig. 4). Unlike the Hus1−/− p21−/− cells, where we observed a 2-fold increase in Chk1 phosphorylation on Ser-345 in the soluble nuclear fraction following HU; we did not observe an increase in Ser-345 phosphorylation in the Hus1−/− p21−/− cells following HU and UV as previously described (26) (Fig. 4A, right panel). However, we detected higher levels of phospho-Ser-345 signal in untreated Hus1−/− p21−/− cells, which had not been described. The discrepancy in our results with the anti-Ser-345 antibodies could be because of the detection assay used (see “Discussion”). We also detected higher levels of phosphorylation of Chk1 on Ser-317 in the chromatin fraction of Hus1−/− p21−/−, whereas the checkpoint-induced phosphorylation of Chk1 on Ser-317 in wild-type cells occurred in both the soluble nuclear and chromatin fractions. This could be because of accumulation of DNA damage in Hus1−/− p21−/− cells related to loss of Hus1 function (26).

These data indicate that the role of Hus1 in the phosphorylation of Chk1 in response to damage and replication blocks is not through regulation of Chk1 chromatin association. The role for the Rad9-Hus1-Rad1 complex could be to provide a platform for Atr and Chk1 to interact at the site of DNA damage/lesion processing. There is residual Chk1 phosphorylation in cells lacking Hus1 or Rad17, suggesting that additional complexes can mediate Chk1 activation by Atr or possibly Atm. Our data also suggest that there are additional components that target Chk1 to DNA.

Claspin, a vertebrate protein that associates with XChk1, plays a role in the activation of Chk1 during DNA replication stress (29), which makes it a candidate protein to regulate chromatin association or activation of human Chk1 during S phase. To test this, we examined the chromatin association and phosphorylation of Chk1 in cells that received either a control morpholino oligonucleotide or antisense morpholino oligonucleotide predicted to interfere with claspin translation. We used a peptide antibody raised against human claspin to detect claspin protein levels, and we monitored the morphology of cells following treatment with HU. The number of cells with abnormal DNA (blebbing) following treatment with HU was higher in cells that had the claspin antisense delivered (22.4%) compared with cells that received the control oligo (3.8%, data not shown). In addition, 3 different peptide antibodies against claspin were found to recognize a protein that migrated as a protein of >175 kDa instead of the predicted 151 kDa. This is similar to what has been observed for Xenopus claspin (29). The protein recognized by these antibodies was significantly reduced in HeLa cells 41 h after delivery of the antisense morpholino (Fig. 4B). Chk1 association with chromatin did not decrease considerably in the cells treated with the antisense morpholino (Fig. 4B); but there was a decrease in the chromatin-associated phosphorylated form of Chk1 following HU or UV after the delivery of the antisense compared with control oligos (Fig. 4B). However, the soluble nuclear phosphorylated form of Chk1 following both treatments in the antisense samples was increased. These data suggest that claspin is not required for the increased phosphorylation of Chk1 following treatment with HU and UV, but it could have a role in retaining the phosphorylated form of Chk1 on chromatin. It is also possible that reducing the levels of claspin leads to the activation of a DNA damage response and hyperphosphorylation of Chk1 via different adaptor molecule (30).

**Ser-345 Is Required for Nuclear Retention of Chk1 following a Checkpoint Signal**—Because Ser-345 phosphorylation was not required to regulate chromatin association of Chk1, it was likely that this phosphorylation regulated an event after chromatin association. The phosphorylation of Ser-345 could function to sequester the checkpoint signal in the nucleus. To address the role of Ser-345 phosphorylation in the nuclear...
localization/retention of Chk1 following a checkpoint signal, we examined the cellular distribution of an EYFP-Chk1S345A mutated protein. We determined that EYFP-Chk1S345A was a functional protein by assaying \textit{in vitro} the phosphorylation of a fusion protein containing Ser-216 of Cdc25C. Ser-345 phosphorylation was not required for Chk1 kinase activity toward Cdc25C, as previously shown (24) (Fig. 5A). The mutation at Ser-345 caused Chk1 to be localized to both the nucleus and cytoplasm even in the presence of a checkpoint signal (Fig. 5, B and C). In addition, mutation of Ser-317 also caused Chk1 to be localized to both the nucleus and cytoplasm in the presence of a checkpoint signal (Fig. 5C), suggesting that the nuclear retention of Chk1 was achieved through phosphorylation of Ser-317 and Ser-345.

There are at least two explanations for these observations. First, Chk1 is shuttled into and out of the nucleus, and upon a phosphorylation signal; a nuclear export signal is masked or inactivated leading to nuclear retention of Chk1. A second possibility is that phosphorylation of Ser-345 causes a conformational change of Chk1 that uncovers a stronger nuclear localization signal. Upon analysis of the Chk1 sequence, an obvious nuclear export signal was not detected. So, we decided to test the former hypothesis by examining Chk1 localization following treatment with Leptomycin B, which blocks Crm1-mediated nuclear export (31).

Treatment of cells expressing EYFP-Chk1 with 2 nM Leptomycin B for 4 h led to accumulation of Chk1 in the nucleus, mimicking the effect of HU or MMS treatment (Fig. 5B). Although treatment of cells expressing EYFP-Chk1 and EYFP-Chk1S345A with HU, Leptomycin B (LMB), or HU + Leptomycin B. The EYFP fusion proteins were visualized by fluorescence microscopy. C, graph depicting the distribution of EYFP-Chk1 and EYFP-Chk1S345A, EYFP-Chk1S317A, and EYFP-Chk1S317A/S345A signals following treatment (+) or not (−) with HU. Purple bars represent the percentage of cells with little difference between nuclear and cytoplasmic EYFP signal (N = C); maroon bars represent cells with the majority of the signal localized to the nucleus (N > C), and the pale yellow bars represent the percentage of cells with EYFP signal in the nucleus only (N). Each bar represents the average of two independent experiments.
Fig. 6. Identification of hydrophobic residues surrounding Ser-345 that could act to mediate nuclear export. Graphical representation of Chk1 protein domains. Shown are the conserved non-catalytic domains that contain Atr phosphorylation sites (SQ1, Ser-317, and SQ2, Ser-345) and the conserved box with MTRF and GD residues. NLS, predicted nuclear localization signal for Chk1 (46). Red circles, residues that...
grown with or without HU. Following HU treatment, a greater percentage of the Chk1M353A protein displayed nuclear retention than the wild-type protein (Fig. 6, A and B, bars labeled \( N > C \) and \( N \)). The Chk1M353A proteins showed a residual cytoplasmic signal and Leptomycin treatment resulted in the M353A mutant localizing exclusively in the nucleus, indicating that there are additional residues (besides Met-353) involved in nuclear export of Chk1. Consistent with this explanation, we observed the same effect as M353A on the cellular distribution of Chk1 with the P344A mutation, however, the EYFP signal was weaker in cells transfected with this construct.\(^7\) The M353A mutation acted in a dominant fashion to the S345A mutation, restoring nuclear retention following a checkpoint signal similar to what had been observed with the wild-type protein (Fig. 6, A and B). These data indicate that residues adjacent to Ser-345 were involved in nuclear export and also support a role for Ser-345 in the retention of Chk1 in the nucleus.

**Chk1 Interaction with 14-3-3 and Crm1**—14-3-3 recognition sites surround phosphoryserine residues (33), and in the case of Cdc25C, phosphorylation of serine 216 (buried within a 14-3-3 recognition motif) correlates with 14-3-3 binding and inactivation (8, 34). The Chk1 proteins have consensus 14-3-3 binding sites COOH-terminal to their kinase domain (amino acids 314–345) (33, 35). In particular, Ser-345 is surrounded by a putative 14-4-3 binding site, and we identified 14-3-3 \( \zeta \) and \( \beta \) in an interaction screen with phosphorylated Chk1,\(^7\) thus 14-3-3 is a candidate protein involved in the nuclear retention of Chk1.

We had previously observed that Chk1 proteins obtained from insect cells and incubated under phosphorylation permissive conditions, would become phosphorylated on Ser-345 and be able to interact with 14-3-3 proteins expressed in bacteria.\(^7\) We examined the association of Chk1 with 14-3-3 and the requirement of Ser-345 for this interaction by pull-down analyses. GST and GST-14-3-3 \( \zeta \) were incubated under phosphorylation permissive conditions with cell lysates prepared from insect cells expressing human Chk1 and mutated Chk1 proteins. Wild-type and Chk1\(^{M353A}\) were pulled down by 14-3-3 (Fig. 6C). A deleted Chk1 missing the COOH-terminal 115 amino acids 361–476, Chk1A, was also found to complex with 14-3-3\(^{3}\) indicating that the region that mediates interaction with 14-3-3 resides in the first 460 amino acids. A mutation at Ser-345 reduced the ability of Chk1 to interact with 14-3-3 (Fig. 6C). Because Chk1S345A also failed to be retained in the nucleus following DNA damage, the association of 14-3-3 with Chk1 is strongly correlated with interference with nuclear export of Chk1. Recently, it has been shown that Chk1 from S. pombe interacts with 14-3-3 via phosphorylation of Ser-345 (36).

If Chk1 is exported via a Crm1 mechanism, then Chk1 should associate with the complex containing Crm1. To address this, we incubated GST and GST-Chk1 fusion proteins with whole cell extracts in the presence of 2 m\( \mu \)GTP and exogenous Ran-GTP (37). The protein complexes were processed by Western analyses with an anti-Crm1 antibody. Chk1 interacted with Crm1 protein (Fig. 6D), and mutation of both phosphoserines in the Atr consensus sites increases the amount of Crm1 that is pulled down by equivalent amounts of Chk1S417A/S345A. Consistent with this, the Chk1S317A also failed to localize to the nucleus following treatment with HU (Fig. 5C). To determine whether Chk1 could form a complex with Crm1 in cells, we precipitated immune complexes from cells expressing FLAG-Chk1 following treatment or not with HU. The FLAG-Chk1 precipitates were examined for the presence of Crm1 by Western analyses. We observed Crm1 association with FLAG-Chk1 in the nuclear extracts obtained from the cells treated or not with HU (Fig. 6E). Although these studies do not establish a direct interaction between Chk1 and Crm1 or 14-3-3, the interaction of Chk1 with Crm1 supports our findings that Leptomycin B can restore nuclear retention to a Ser-345-mutated Chk1 by blocking its export. Thus, phosphorylation of Ser-345 could serve as a docking site for 14-3-3 proteins that would lead to the interference with a nuclear export signal present in a region surrounding Ser-345.

**DISCUSSION**

**Signaling Molecules Associated with Chromatin**—Like Atr, we found that Chk1 is associated with chromatin in the absence of an induced checkpoint signal. The proportion of Chk1 phosphorylated on Ser-345 was increased in both the soluble nuclear and chromatin pools of Chk1 following a checkpoint signal. However, S345A phosphorylation-defective Chk1 was also found to be associated with chromatin, supporting the argument that the phosphorylation of Ser-345 is not required for chromatin localization, and that the phosphorylation event is likely to take place on chromatin.

Thus, the DNA damage-stimulated phosphorylation of Chk1 on Ser-345 seems to be required for a step following localization of Chk1 to chromatin, perhaps when the checkpoint kinase-containing complexes and the sensor complexes come together on DNA. Subsequent phosphorylation of Chk1 leads to binding and phosphorylation of other proteins for signaling to downstream targets such as Cdc25C (7, 8) and p53 (12).

We observed low levels of Ser-345-phosphorylated Chk1 in untreated cycling cells. This is likely because of a checkpoint signal activated during every S phase. The low level of phosphorylation could also be because of the fact that the cells in these experiments are derived from a genetically unstable neo-
plasm, which could have constitutive low level checkpoint signals. An argument against the latter is that phosphorylated Chk1 was present in the chromatin fraction of early passage mouse embryonic fibroblasts as well.

How does the signal from the chromatin-associated Chk1 reach effectors such as Cdc25C? One model would propose that the chromatin-associated Chk1 becomes phosphorylated, at which point it is released from the chromatin fraction and is exported from the nucleus. This would allow 14-3-3 to bind to the phosphorylated Chk1 in the cytoplasm, and following nuclear re-entry, the 14-3-3-bound form would be retained. Consistent with this, we detected phospho-Chk1Ser-317 in the cytoplasmic fraction from treated cells (Fig. 3, A and C). Alternatively, a very small pool of 14-3-3 proteins present in the nucleus could bind to the phosphorylated Chk1 (38).

14-3-3 and Checkpoint Signaling—The role of 14-3-3 proteins in both checkpoint signaling and nuclear-cytoplasmic shuttling has been documented for proteins such as p53 and Cdc25 (38, 39). 14-3-3 β and ζ interact with the phosphatase Cdc25 that has been phosphorylated on Ser-216, and this is proposed to be a mechanism for checkpoint-induced inactivation or nuclear export of this Cdk/cyclin activating enzyme (40). 14-3-3 σ, a transcriptional target of p53, does not interact with Cdc25 but is required for the maintenance of the G1/M checkpoint (41). In addition, fission yeast Chk1 association with 14-3-3 proteins has been shown to be stimulated by DNA damage (35).

Here we provide evidence that the mammalian Chk1 kinase interacts with 14-3-3 and that the nuclear retention of Chk1 is regulated by phosphorylation at a putative 14-3-3 binding site. Chk1 contains a second potential 14-3-3 binding site surrounding an Atr/Atm consensus phosphorylation site NH2-terminal to Ser-345 (Ser-317 within amino acids 314–319), which could provide tighter binding to a 14-3-3 dimer and thus contribute to the regulation of Chk1 (42). 14-3-3 binding could also have a role in maintaining the kinase in an “active” conformation by disrupting the inhibitory effect proposed for the COOH-terminal tail (43), or mediating interaction with its substrates. The fact that the mutation of Ser-317 caused an increased interaction of Chk1Ser-345 with Crm1 in our pull-down assay suggests that phosphorylation of both serines cooperate to regulate the localization of Chk1.

The observations described here for human Chk1 are different from those observed with a Chk1Ser-345 mutant in the fission yeast where the cellular distribution was reported to be the same as that of the wild-type protein (25). This difference could be because of a role for the activated form of mammalian Chk1 in the initial and transient signaling to cytoplasmic effectors that was not conserved in the S. pombe protein.

The Sensor Complexes and Adapter Molecules—Rad17 is required for Rad9-Hus1-Rad1 to be loaded on chromatin, and Hus1 is required for the Rad17 phosphorylation by the Atr-containing complexes. Both the clamp loader, hRad17, and clamp Rad1-Hus1-Rad9 sensor complexes play a role in the Atr-mediated phosphorylation of Chk1. However, Hus1 is not required for the association of Chk1 with chromatin, which suggests that there are additional proteins involved in mediating the interaction between the checkpoint kinase-containing complexes to initiate a signaling cascade. We observed a higher basal level of Ser-345 phosphorylation in Hus−/−p21−/− cells, which is different from published results. The difference between ours and published results could be because of the detection method, here we have analyzed denatured proteins by Western analyses, whereas previous studies used immunoprecipitation of phospho-Chk1 coupled with Western analyses with an anti-Chk1 antibody. It is likely that interaction between phosphorylated Chk1 and other proteins could interfere with antibody binding. In addition, the phosphorylation of Chk1 on Ser-317 in response to HU and UV is enhanced in the Hus−/− p21−/− cells, indicating players other than the Hus1 complex in the phosphorylation of Chk1.

One possible player in this response is claspin; a Chk1-interacting protein identified in Xenopus laevis and is required for XChk1 activation by the replication checkpoint (29). The yeast orthologue of claspin (Mrc1) has been proposed to act as an adaptor molecule that could bind phosphorylated checkpoint kinases and promote amplification of the checkpoint signal. Budding yeast have two such molecules, Rad9 and Mrc1, which function in the DNA damage and S phase checkpoint, respectively (30, 44, 45), thus it is likely that amplification of the checkpoint signal caused by the lesions in the cells treated with claspin antisense is because of interaction of Chk1 with another protein with a similar role.

A Role for Chk1 in the S Phase Checkpoint—The intra-S phase checkpoint is required for the regulation of replication origin stability and recovery, and to prevent the firing of late replication origins. In S. cerevisiae, this is the role of the Rad53 kinase (hChk2 homologue). Chk1 is associated with chromatin and phosphorylated on Ser-317 and Ser-345 even in the absence of exogenous checkpoint signals. This is consistent with a role for Chk1 in the checkpoint that monitors replication fork stability and replication blocks.

The successful duplication and segregation of genetic information requires many layers of regulation of the proteins that control the cell cycle. Similarly, checkpoint proteins that ensure the completion of cell cycle events and genomic integrity are also tightly regulated. Here we provide evidence for at least two steps of regulation of the checkpoint kinase Chk1. Chk1 is associated with chromatin, and following a checkpoint signal (endogenous or exogenous) becomes phosphorylated on Atr phosphorylation sites. Phosphorylation on Ser-345 occurs in a region that mediates both 14-3-3 binding and nuclear export. Our data are consistent with a model where phosphorylation of Chk1 on Ser-345 (and possibly Ser-317) leads to binding by 14-3-3, which masks residues that would mediate nuclear export, and thus keep the checkpoint signal localized in the nucleus. Whether the activated chromatin-bound Chk1 has a role in DNA repair and whether 14-3-3 binding also facilitates interaction of Chk1 with its substrates remains to be determined.

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Regulation of Chk1 Includes Chromatin Association and 14-3-3 Binding following Phosphorylation on Ser-345
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