DNA Damage Regulates Chk2 Association with Chromatin*

DNA damage triggers cellular signaling pathways that control the cell cycle and DNA repair. Chk2 is a critical modulator of diverse responses to DNA damage. Chk2 transmits signals from upstream phosphatidylinositol 3-kinase-like kinases to effector substrates including p53, Brca1, Cdc25A, and Cdc25C. Using chromatin fractionation as well as immunostaining combined with detergent pre-extraction, we have found that a small pool of Chk2 is associated with chromatin prior to DNA damage. Recovery of chromatin-bound Chk2 is reduced in an ATM-dependent manner by exposure to ionizing radiation. Camptothecin and adriamycin also reduce the amount of chromatin-associated Chk2. The Thr68-phosphorylated forms of Chk2 induced by DNA damage are found in soluble fractions, but not in the chromatin-enriched fraction. Functional serine/threonine glutamine cluster domain, forkhead-associated domain, and kinase activity are all required for efficient reduction of chromatin-bound Chk2 in response to DNA damage. Artificial induction of Chk2 oligomerization concomitant with exposure to low dose ionizing radiation reduces chromatin-bound Chk2. When Chk2 is incubated with chromatin-enriched fractions in vitro in the presence of ATP, hyperphosphorylated forms of Chk2 bind more weakly to chromatin than hypophosphorylated forms. Taken together, our data suggest that DNA damage induces activation of chromatin-bound Chk2 by a chromatin-derived signal, and that this results in dissociation of activated Chk2 from chromatin, facilitating further signal amplification and transmission to soluble substrates.

Genomic insults from intrinsic factors, such as DNA replication errors and products of oxidative metabolism, or exogenous factors, including genotoxic chemicals, ultraviolet light, or ionizing radiation (IR), pose constant threats to eukaryotic cells. DNA damage checkpoint pathways sense the damage promptly and execute coordinated cellular responses, including cell cycle arrest to allow time for efficient recovery, transcriptional induction of repair-related genes, and/or apoptosis. DNA damage checkpoint pathways are required for the maintenance of genome stability and prevention of tumor development.

ATM and ATR, members of the phosphatidylinositol 3-kinase-like kinase (PIKK) family, play central roles in damage responses. ATM is activated mainly by DNA double strand breaks (DSBs). Cells from patients with ataxia telangiectasia (AT), a severe disease caused by ATM mutations, have IR hypersensitivity, and defects in IR-induced G1, S, and G2/M cell cycle arrest. ATR responds to a broader range of signals, including UV-induced damage, DSBs, and stalled replication forks (1). ATM and ATR phosphorylate a broad spectrum of substrates, including Chk1 and Chk2, which are important downstream effector kinases with overlapping functions.

The principal kinase that relays signals initiated by ATM appears to be Chk2. UV irradiation or replication blockade cause the phosphorylation of Chk2 independent of ATM, possibly through ATR (2, 3). Activated Chk2 mediates the rapid IR-induced G1 arrest and inhibition of DNA synthesis through the phosphorylation of Cdc25A, which triggers the ubiquitination and proteosomal degradation of Cdc25A (4). In addition, Chk2 participates in G2/M arrest through the phosphorylation of mitosis-promoting Cdc25C phosphatase (5). By phosphorylating p53, Chk2 also helps maintain sustained G1, G2/M arrest, and apoptosis (6). Additional substrates of Chk2 include the tumor suppressor Brca1, PML, and the transcription factor E2F1 (7–9).

Function of Chk2 requires several evolutionarily conserved domains. They include an amino-terminal SCD (SQ/TQ cluster domain), which contains multiple preferred SQ/TQ phosphorylation sites for PIKKs, a FHA (forkhead-associated) domain, which binds to phosphopeptides, and a carboxyl-terminal protein kinase domain (6). ATM phosphorylates Chk2 at Thr68 (2, 3), which is followed by oligomerization of Chk2 through FHA domain/phospho-SCD interactions, autophosphorylation, and activation (10–12). Phosphorylation of Thr68, located in the SCD, and the integrity of the FHA domain are required for full activation of Chk2 (2, 3, 12, 13).

DNA damage checkpoint cascades involve the dynamic relocation of DNA damage signaling and repair proteins. Various techniques, including fluorescent tagging, immunofluorescence, chromatin immunoprecipitation, and biochemical fractionation have revealed the damage-induced spatiotemporal behavior of checkpoint proteins. In Saccharomyces cerevisiae, the Ddc1-Rad17-Mec3 complex (Rad9-Rad1-Hus1 homologs) and Rad24 (Rad17 homolog) are recruited to the DNA damage sites through a distinct mechanism from the recruitment of Mec1-Ddc2 (ATR-ATRIP homologs) (14, 15). Similarly, in mammalian cells, Rad17 (a RFC-like protein) binds to chromatin prior to damage and, after DNA damage recruits Rad9-Rad1-Hus1, a proliferating cell nuclear antigen-like protein complex, whereas ATR-ATRIP are recruited to chromatin independently. The chromatin-bound 9-1-1 complex helps ATR recruit and phosphorylate its substrates, including Rad17 (16). Replication protein A (RPA)-coated single-stranded DNA facilitates the recruitment of both ATRIP-ATR and Rad17/Rad9-Rad1-Hus1 complexes to DNA damage sites or stalled replication forks (17, 18). Exposure to IR, which induces various DNA lesions including DSBs, results in accumulation and phosphorylation of proteins to include the phospho-isoform γ-H2AX (19), ATR (20), members of the MRN (Mre11-Rad50-Nbs1) complex (21), phosphorylated ATM and DNA-PKCs (22, 23) at discrete foci that are located at sites of DNA damage and repair. DNA damage causes the retention of ATM, Rad9, and 53BP1...
in detergent extraction-resistant fractions (24–26). A recent temporal study reported that, in budding yeast, Mre11 recruits the ATM homolog Tel1 to DSBs, which precedes and is independent of the recruitment of RPA, Rad24, and Ddc1-Rad17-Mec3. Later, DNA repair proteins assemble at the DSBs (27).

Immunodetection of the bulk population of Chk2 has not revealed damage-regulated relocalization of Chk2 (28, 29). Based on findings for checkpoint proteins ATM and 53BP1 (24, 26), we hypothesized that the soluble nuclear Chk2 pool masks a fraction of Chk2 whose chromatin association is regulated by DNA damage. We have used chromatin fractionation and in situ detergent pre-extraction combined with immunofluorescence to investigate dynamic regulation of Chk2 by DNA damage. We found that a small pool of Chk2 is associated with chromatin before DNA damage. Irradiation reduces chromatin-bound Chk2 in an ATM-dependent manner. Topoisomerase inhibitors also decrease the association of Chk2 with chromatin. Hyperphosphorylated Chk2 binds more weakly to chromatin than its hypophosphorylated counterpart in vitro.

MATERIALS AND METHODS

Cell Culture and Chromatin Fractionation—Immortalized A-T fibroblasts A-T221JE-T stably transfected with pEB57 encoding full-length ATM tagged with FLAG or the control vector were kindly provided by Y. Shiloh and M. Kastan (30). HEK293 and AT cells stably transfected with vector or wild type ATM were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, l-glutamine, and antibiotics.

Chromatin fractionation was carried out as described in Ref. 31 with some modifications. Cells were washed twice with phosphate-buffered saline (PBS) (Ca2+/Mg2+/free) and resuspended in solution A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10 mM HEPES, pH 7.5, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol), in the presence or absence of an ATP-regenerating system (5 mM ATP, 70 mM creatine phosphate, 0.1 mg/ml creatine kinase). Insoluble chromatin and proteins remaining in the supernatant were separated by centrifugation. The chromatin pellet was washed with solution A and the wash was combined with the supernatant. SDS sample buffer was added to the chromatin pellet. For FLAG-Chk2, the supernatant fraction was mixed with 200 μl of NETN buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor), and anti-FLAG M2 affinity beads (Sigma) were added. Immunoprecipitations were performed for 3 to 4 h. Precipitates were washed four times with NETN buffer. Then, SDS sample buffer was added to the beads. For GST-Chk2, SDS sample buffer was added directly to the supernatant.

For consecutive chromatin binding and release assays (Fig. 8C), GST-Chk2 was incubated for 40 min at 30 °C with the HEK293 chromatin preparation in kinase buffer A, in the absence of an ATP-regenerating system. The reaction mixture was separated into three aliquots. No further incubation was carried out on the first aliquot. The second aliquot was incubated for another 40 min at 30 °C. An ATP-regenerating system was added to the third aliquot, before it was further incubated for another 40 min at 30 °C. For all three samples, insoluble chromatin and proteins remaining in the supernatant were separated by centrifugation. The chromatin pellet was washed once with solution A and the wash was combined with the supernatant. SDS sample buffer was added to the chromatin pellet and the supernatant.

Transfection and Experimental Treatments—Cells were transfected with FuGENE 6 (Roche). Stable transfectants were selected in growth medium containing G418 (Invitrogen). Cells were irradiated in a Mark I 137Cs irradiator (Shepard), treated with 1 μM camptothecin (Sigma) or 2 μM adriamycin (Sigma). For UV treatment, cells were irradiated with 254-nm UV light at a dose of 50 J/m2 with a Stratalinker (Stratagene).

Recombinant Protein Purification—Expression of GST-Chk2 in E. coli strain BL21 was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h. Cells were resuspended in lysis buffer (PBS, 1% Triton X-100, 10% glycerol, protease inhibitor) with 0.5 mg/ml lysozyme, and sonicated. Glutathione-Sepharose beads (Amersham Biosciences) were added to cleared lysates, and the mixture rotated for 2–4 h or overnight at 4 °C. The beads were washed four times with lysis buffer. GST fusion protein was eluted with Glutathione Elution Buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione).

Induced Oligomerization of 2FKBP-Chk2—HEK293 cells stably transfected with pcDNA-HA-2FKBP-Chk2 (WT or kinase-defective) (described in Ref. 10) were either mock-treated or exposed to 2.5 Gy of γ-irradiation immediately before the addition of 10 nM of the bivalent ligand AP20187 (Ariad Pharmaceuticals) or ethanol as vehicle control. Cell lysates were prepared and fractionated at the indicated times.
Chk2 Association with Chromatin

Immunofluorescence—HEK293 cells were grown on 8-well culture slides (BD Biosciences). Cells were treated, or not, with 1 μM camptothecin for 3 h. Cells were washed twice in PBS, before being pre-extracted in situ on ice for 5 min with 0.1% Triton X-100 in 10 mM HEPES (pH 7.4), 2 mM MgCl2, 100 mM KCl, and 1 mM EDTA. Cells were then fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized in 0.5% Triton X-100 in PBS for 15 min at room temperature. Cells were stained with polyclonal anti-Chk2 antibody (Santa Cruz) diluted in blocking buffer (2% bovine serum albumin, 0.1% Tween 20/PBS) at 37 °C. The slides were mounted with Anti-fade mounting medium containing 4’,6-diamidino-2-phenylindole (Molecular Probes). Images were captured using an Olympus BX51 microscope equipped with a Sensicam camera.

RESULTS

Chromatin-bound Chk2 Level Is Decreased after Certain Types of DNA Damage—Chk2 is phosphorylated and activated in response to DNA damage and replication blockade. Because Chk2 is important for the transduction of DNA damage signals, we investigated if Chk2 associates with chromatin and whether it undergoes damage-regulated relocalization. Cellular extracts were fractionated into cytoplasmic (S1), soluble nuclear (S2), and chromatin-bound components (P) (16, 31). Grb2 and Orc2, used as markers for S1 and P, respectively, were resolved well when these fractions were prepared from HEK293 cells (Fig. 1A). Grb2 and Orc2 levels were not altered when cells were exposed to γ-irradiation prior to lysis (Fig. 1A, lanes 3–6). Chk2 is a nuclear protein (7, 8, 28, 29). There was substantial recovery of Chk2 in cytosolic fractions (Fig. 1B, lane 7), presumably originating in leakage from a soluble nuclear compartment. The clean fractionation of Orc2 suggests that chromatin-bound proteins will not be affected under these conditions. A small proportion of Chk2 was detected in the chromatin-enriched fraction (P) in cells undergoing unperturbed growth (Fig. 1B, lane 1). This component was reduced by treatment with micrococcal nuclease (MNase) (Fig. 1C), confirming that this material is associated with chromatin. Under these conditions, most of the chromatin-bound Orc2 was released by MNase into soluble fractions (Fig. 1C).

To determine whether DNA damage affects the distribution of chromatin-bound Chk2, HEK293 cells were exposed to either IR or UV before fractionation. IR induced the electrophoretic mobility shift associated with Chk2 hyperphosphorylation (Fig. 1B, lanes 5 and 8). Interestingly, IR, but not UV, reduced the proportion of Chk2 associated with chromatin (Fig. 1B, lanes 1–3). Chk2 levels in cytosolic fractions (S1) or whole cell lysates did not change after DNA damage (Fig. 1B, lanes 7–9, and data not shown). Pretreatment of cells with the proteasome inhibitor MG132 had no effect on the decrease in chromatin-bound Chk2 (data not shown). The IR-induced hyperphosphorylated form of Chk2, which is the active form, was found mostly in the soluble fractions, including cytoplasmic and soluble nuclear fractions, but not in the chromatin-enriched fraction (Fig. 1B, compare lanes 2, 5, and 8). Chromatin association of Orc2, the marker for the chromatin fraction, was not altered by either IR or UV treatment (Fig. 1B, lanes 1–3). Similar results were obtained with IR-treated U2OS cells (data not shown). Increasing doses of IR progressively reduced the amount of chromatin-associated Chk2 relative to the S1 fraction (Fig. 2, upper panel). This reduction of chromatin-associated Chk2 occurs rapidly, within 10 min after 10 Gy of IR (Fig. 2, middle panel). The hyperphosphorylated form of Chk2 appeared rapidly in both S1 and S2 fractions after 2 Gy of IR, but there was little change in the amount of chromatin-associated Chk2 (Fig. 2, bottom panel). Because the functional activation of nuclear ATM precedes and is separable from its retention at DNA double strand breaks (35, 36), it is possible that the activated nuclear ATM also phosphorylates and activates soluble nuclear Chk2.

Similarly, pre-treatment of cells with adriamycin or camptothecin for various lengths of time resulted in reduction of chromatin-associated Chk2 concomitant with the hyperphosphorylation (mobility shift) of Chk2 in the soluble fractions (Fig. 3). Phosphorylation of Chk2 at Thr68 is a prerequisite for the activation of Chk2 by IR (2, 3, 12, 13). The Thr68-phosphorylated Chk2 form was observed in the soluble fractions, but was not detected in the chromatin-enriched fractions (Fig. 3). In all conditions that induced Chk2 phosphorylation, IR, adriamycin, or camptothecin treatment (Figs. 1B and 3), loss of Chk2 from the chromatin fraction correlated well with the hyperphosphorylation of Chk2 in other fractions, suggesting that phosphorylation is required to release
Mutations in Chk2 Affect Reduction of Chromatin-associated Chk2—One interpretation of the foregoing results is that Chk2 activation is required for its dissociation from chromatin after DNA damage. Activation of Chk2 requires the Chk2 FHA domain, phosphorylation of one or more sites in the SCD, including Thr68, and autophosphorylation of one or more sites in the catalytic domain. The function of conserved domains of Chk2 was studied by using stable HEK293 cell lines expressing endogenous Chk2 and various exogenous FLAG-Chk2 mutants (Fig. 6). FLAG-tagged full-length Chk2 molecules are distinguished from endogenous Chk2 by their greater size, so that behavior of FLAG-tagged exogenous and endogenous WT Chk2 could be compared directly in the same cells. Exogenous WT FLAG-Chk2 behaved similarly to endogenous Chk2 in IR-induced reduction in chromatin fractions (Fig. 6B, lane 1 and 2). Kinase-defective and T68A Chk2 mutants were also similar to endogenous Chk2, although their greater expression may have masked a small change in association after IR (Fig. 6B, lanes 5–8). In contrast, IR did not reduce chromatin association of Chk2 lacking the entire SCD (Chk2ΔSCD) (Fig. 6B, lanes 9 and 10). The FHA domain, a phosphopeptide binding motif, is important for Chk2 Thr68 and activation loop autophosphorylation in vivo (12). Substitution of the conserved residues NGT-(166–168) with alanines in the Chk2NGT mutant, or deletion of the core FHA domain, substantially decreased its in vitro kinase activity (10). Chromatin association of Chk2NGT or Chk2ΔFHA mutants was not affected by IR (Fig. 6B, lanes 3, 4, 11, and 12).
J2). Thus the SCD and FHA domains, which are all essential for Chk2 activity, are important for the IR-induced release of Chk2 from chromatin. We have shown that single point substitution of the SCD SQ/TQ sites with alanine, including T68A, does not significantly affect Chk2 oligomerization (10). This is consistent with our current finding that chromatin-associated Chk2-T68A was reduced similarly to wild type Chk2 after IR. Chk2-T68A has a similar mobility to its wild type counterpart in both damaged and undamaged nuclear regions in fluorescence loss in photobleaching assays (29).

Chk2 oligomerization mediated by interaction of the FHA domain with the phosphorylated SCD is important for Chk2 activation and signal transduction (10, 11). Oligomerization of Chk2 can also be induced artificially by fusing Chk2 to two tandem dimerization modules (FKBP[F36V]) that are then induced to oligomerize by the addition of the cell-permeable, synthetic ligand AP20187 (10). In our earlier work, induced oligomerization enhanced Chk2 Thr68 phosphorylation in vivo and facilitated Chk2 activation after low dose IR treatment, as demonstrated by increased Chk2 autophosphorylation and phosphorylation of the substrate Cdc25C (10). To determine the effects of Chk2 oligomerization on chromatin release, HEK293 cells stably transfected with HA-tagged 2FKBP-Chk2 were treated with 2.5 Gy of IR. Addition of the ligand AP20187 immediately after IR exposure resulted in a reduction of the chromatin-bound wild type Chk2 level at early time points (Fig. 7A, lanes 3–10), and concomitantly increased phosphorylation of Chk2 at Thr68 as previously shown (Ref. 10, and data not shown). Our ability to complement low doses of IR with the forced oligomerization signal, which alone fails to cause Chk2 dissociation from chromatin (data not shown), suggests that transphosphorylation of Chk2 is a necessary priming event for the release of Chk2. In contrast, addition of the ligand after IR treatment had no apparent effect on the recovery of chromatin-bound kinase-defective Chk2 (Fig. 7B). Apparently, the failure of kinase-defective Chk2 to trans-autophosphorylate after forced oligomerization of kinase-defective Chk2 molecules contributes to the lack of damage-induced dissociation. Kinase-defective Chk2 did dissociate from chromatin after IR (Fig. 6B, lanes 7 and 6). This may be caused by oligomerization of kinase-defective Chk2 with endogenous wild type Chk2 (10), leading to partial trans-autophosphorylation and dissociation from chromatin. Overall, phosphorylation of Chk2 by upstream kinase(s), oligomerization, and autophosphorylation are associated with the loss of Chk2 from chromatin.

Binding of Chk2 to Chromatin in Vitro—Our in vivo experiments suggested that chromatin-bound Chk2 is activated and then dissociates...
from chromatin, possibly allowing further activation of downstream effectors or other inactive Chk2 molecules by activated Chk2. We developed an in vitro assay to study the chromatin binding properties of hypophosphorylated and hypophosphorylated Chk2. Chk2 produced in vitro using a wheat germ-derived translation system is not hyperphosphorylated, lacks Thr207/Ser208 and Thr68 phosphorylation, and has minimal autophosphorylation activity (10). Incubation of hypophosphorylated wheat germ-translated Chk2 with rabbit reticulocyte lysates enhances Thr68 phosphorylation and kinase activity (10). Incubation of wheat germ-translated hypophosphorylated Chk2 with cytoplasmic, soluble nuclear, or chromatin fractions from HEK293 cells in the presence of an ATP-regenerating system resulted in Thr68 phosphorylation, with the most efficient phosphorylation of Chk2 occurring after incubation with the chromatin-enriched fraction (37). The Thr68 phosphorylation of Chk2 by the chromatin fraction was greatly diminished by preincubation of chromatin with wortmannin (37), a specific PIKK inhibitor (38), indicating that ATM, ATR, and/or DNA-PK are likely to be the kinases responsible for the phosphorylation of Chk2 in this in vitro assay.

To determine whether the hypophosphorylated and hyperphosphorylated Chk2 differ in their abilities to bind chromatin, wheat germ-translated Chk2 was incubated with the non-soluble chromatin fraction in the absence or presence of ATP. The soluble and non-soluble portions were then separated by centrifugation. The supernatant, containing Chk2 that did not bind chromatin, and pellet, containing chromatin and chromatin-bound Chk2, were compared for Chk2 abundance and phosphorylation. Incubation of Chk2 with chromatin fractions from either irradiated or untreated cells yielded similar levels of Chk2 Thr68 phosphorylation (Fig. 8A, compare lanes 1, 2 to 3, 4). The distribution of Chk2 between the supernatant and pellet was also similar after incubation with chromatin fractions from irradiated or untreated cells (Fig. 8A, lower panel, compare lanes 1, 2 to 3, 4). Chk2 incubated with chromatin in the absence of ATP was not phosphorylated, and was recovered preferentially in the chromatin-containing pellet (Fig. 8A, lower panel, lanes 5 and 6). A subset of Chk2 incubated with chromatin fractions in the presence of ATP was phosphorylated on Thr68 (Fig. 8A, lanes 1–4), and Thr68-phosphorylated Chk2 was enriched in the portion of Chk2 recovered in the supernatant after incubation. Correspondingly, the majority of the chromatin-bound Chk2 was not phosphorylated on Thr68, in contrast to the strong phospho-Thr68 signal of Chk2 in the supernatant.

When expressed in bacteria, Chk2 is capable of efficient autophosphorylation in the absence of other eukaryotic proteins (10). GST-Chk2 produced in E. coli is hyperphosphorylated, as indicated by heterogeneously migrating bands on SDS-PAGE, and a strong phospho-Thr68
signal in immunoblots. In contrast, kinase-defective GST-Chk2 is not phosphorylated when expressed in bacteria. When wild type or kinase-defective GST-Chk2 were incubated with the chromatin fraction in the absence of an ATP-regenerating system, then centrifuged, GST-Chk2 were found in both the supernatant and chromatin-containing pellet (Fig. 8B, upper panel, lanes 1, 2, 5, and 6). Incubation with chromatin in the presence of ATP caused further Thr<sup>68</sup> phosphorylation of bacterially produced GST-Chk2 (Fig. 8B, compare lanes 1, 2 to 3, 4). This enhanced phosphorylation was accompanied by near elimination of Chk2 associated with the chromatin-enriched fraction. Kinase-defective GST-Chk2 was phosphorylated in trans by the chromatin fraction in the presence of ATP, and this phosphorylation moderately augmented partitioning of GST-Chk2 to the soluble fraction. Nonetheless, virtually all of the Thr<sup>68</sup>-phosphorylated KD GST-Chk2 was recovered in the soluble fraction (Fig. 8B, lanes 7 and 8).

Taken together, these results suggested either that phosphorylated Chk2 initially associates with chromatin less well than its hypophosphorylated counterpart, or that it associates well but is released more rapidly. In Fig. 8C, WT and KD GST-Chk2 were incubated with chromatin-enriched fractions in vitro in the absence of ATP to promote binding. The reaction mixtures were divided and either fractionated by centrifugation directly or further incubated in the absence of ATP in the presence of ATP, and then fractionated by centrifugation. The greater recovery of GST-Chk2, and its greater phosphorylation on Thr<sup>68</sup> in the supernatant, suggested that Thr<sup>68</sup> phosphorylation enhances release of Chk2 from the insoluble fraction (Fig. 8C, compare lanes 5, 6 to lanes 1, 2). Consistent with in vivo oligomerization data in Fig. 7, the KD Chk2 remained primarily associated with the insoluble pellet when incubated in the presence of ATP (Fig. 8C, lanes 11 and 12). Once again, the Thr<sup>68</sup>-phosphorylated subset was greatly enriched in the supernatant fractions.

**DISCUSSION**

In this study we provide both in vivo and in vitro evidence that a small pool of Chk2 associated with chromatin is activated and released from chromatin in response to DNA damage. Exposure of cells to IR, adriamycin, or camptothecin reduces the recovery of chromatin-associated Chk2 by the chromatin fractionation method (Figs. 1B and 3), which has been corroborated by in situ detergent pre-extraction combined with immunofluorescence (Fig. 4). The IR-dependent decrease of chromatin-associated Chk2 requires ATM (Fig. 5). The hyperphosphorylated Chk2 is found mainly in the soluble fractions, but not in the chromatin fraction (Figs. 1B, 3, and 5). In the in vitro chromatin binding assay, phosphorylation of hypophosphorylated Chk2 by protein kinases present in the chromatin-enriched fraction results in reduced recovery of hyperphosphorylated Chk2 in the chromatin pellet (Fig. 8, A and B). The phosphorylation by the chromatin-enriched fraction also induces the release of previously chromatin-bound Chk2 into the supernatant (Fig. 8C). Other work from our laboratory implicates chromatin-derived wortmannin-sensitive kinase(s), probably ATM and DNA-PK, in this process (37). The release of chromatin-bound Chk2 in vivo is hindered when its SCD, FHA, or kinase domain is mutated (Figs. 6 and 7), which are all essential for the full activation of Chk2. This is further corroborated by in vitro evidence that phosphorylation-induced partitioning of KD Chk2 from the chromatin to supernatant is impaired relative to the WT Chk2 (Fig. 8, B and C). Taken together, these results indicate that the activation of Chk2 by chromatin-associated PIKKs enables the release of Chk2 from chromatin.

Thr<sup>68</sup>-phosphorylated forms of both FLAG-Chk2 produced by in vitro translation, and GST-Chk2, expressed in bacteria, preferentially partitioned to the supernatant after incubation with a chromatin-enriched fraction in the presence of ATP. However, bacterial recombinant Chk2 is highly phosphorylated at Thr<sup>68</sup>, probably because of high level expression and cross-phosphorylation. Hyperphosphorylated bacterial GST-Chk2 behaves differently before and after incubation with chromatin in the presence of ATP (Fig. 8, B and C). This may be because of different conformation or phosphorylation site selections between the two preparations. Or, the difference may reflect an ATP dependence for the release, consistent with the experimental results with WT and KD proteins.

Rapid sensing of DNA damage and the efficient amplification of the initial signal are critical for the proper cellular response. Studies in budding yeast have shown that a single unreparable DSB can trigger a

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**Figure 8**. Chk2 chromatin binding and release in vitro. A and B, FLAG-Chk2 translated from wheat germ extract in vitro (A) or GST-Chk2 (WT or kinase-defective) expressed in E. coli (B) was incubated for 1 h at 30°C with chromatin preparations from irradiated or untreated HEK293 cells (A), or untreated HEK293 cells (B), suspended in kinase buffer A, in the presence or absence of an ATP-regenerating system. Insoluble chromatin and proteins that were not associated with chromatin were separated by centrifugation. C, WT or kinase-defective (kd) GST-Chk2 expressed in E. coli was incubated for 40 min at 30°C with HEK293 chromatin preparation in kinase buffer A, in the absence of an ATP-regenerating system. The reaction mixture was analyzed directly (lanes 1, 2, 7, and 8), or after further incubation for 40 min at 30°C in the absence (lanes 3, 4, 9, and 10) or presence (lanes 5, 6, 11, and 12) of an ATP-regenerating system. Insoluble chromatin and proteins remaining in the supernatant were separated by centrifugation. Phosphorylation of Thr<sup>68</sup> was monitored by immunoblotting with anti-pT68-Chk2 antibody. The membrane was stripped and reprobed with anti-FLAG horseradish peroxidase or anti-Chk2, IB, immunoblot; S, supernatant; P, pellet.
global damage response (39). Recently, evidence has accumulated for the dynamic relocation of DNA damage signaling and DNA repair proteins to sites of DNA lesions. Histone H2AX is rapidly phosphorylated in the chromatin surrounding DSBs by PIKKs including ATM after IR (40). γH2AX (phosphorylated H2AX) is essential for the retention of checkpoint mediators, including BRCT domain-containing 53BP1, Brca1, and MDC1/NFBD1, at the damage sites (41–43). 53BP1, Brca1, or MDC1/NFBD1 regulates Chk2 phosphorylation after DNA damage (44–46). PIKKs, including ATR, autophosphorylated ATM, or autophosphorylated DNA-PK, as well as the DNA damage sensor proteins, such as RPA, Rad17, and Mre11-Rad50-Nbs1 complex, also relocalize to DNA damage sites (16, 18, 21–23). The dissociation from chromatin after DNA damage distinguishes Chk2 from many other DNA damage signaling proteins, and supports the role of Chk2 as checkpoint signal transmitter.

Besides the recruitment of sensor and repair proteins to DNA damage sites, it is of equal importance for the signal to be transduced to the other compartments of the affected cells within a short period of time. Chk2 is a nuclear protein, but the detailed intranuclear localization pattern of Chk2 is controversial. Many studies have aimed to comprehensively catalog the relocation of yeast checkpoint proteins to sites of DNA damage, including the budding yeast Chk2 ortholog, Rad53. Rad53 does not relocalize to foci in _cdc13-1_ cells after growing at non-permissive temperature (14). However, γ-irradiation or hydroxyurea results in focus formation of Rad53-GFP, which is a relatively early event prior to the recruitment of recombination proteins, such as Rad52. It is noteworthy that even under the circumstances wherein Rad53 relocalizes to DNA damage sites, the Rad53 foci are faint, suggesting that Rad53 is not well concentrated at the damage sites (27). Using a chromatin immunoprecipitation method, HU-induced Rad53 association with replication regions is barely detectable even when a protein–protein cross-linking reagent is used before formaldehyde fixation (47).

Likewise, although mammalian Chk2 is a predominantly nuclear protein, descriptions of the subnuclear distribution of Chk2 are inconsistent. In some studies, Chk2 was reported to have a diffuse nuclear immunostaining pattern in untreated or irradiated cells (28, 29), whereas others report that Chk2 exists in distinct nuclear foci before or after DNA damage (7, 8). The reason for the discrepancies is not clear, although the use of different cell lines and antibodies may be important. Some reports indicate that Chk2, phosphorylated at Thr68 in response to DNA damage, localizes to sites of DNA damage (28, 32, 44). However, this has been disputed in another study that raises questions about specificity of the phospho-antibodies, and reports that Chk2 is highly mobile, even after DNA damage (29). Regardless, immunodetection of the bulk population of Chk2, rather than phosphorylated forms, has not revealed damage-regulated relocalization of Chk2 (28, 29). Although co-immunoprecipitation experiments showed that the constitutive association of Chk2 and PML was disrupted by irradiation, Chk2 remains to localize to the PML nuclear bodies after irradiation by immunostaining (8). Because immunostaining and fluorescence loss in photobleaching experiments indicate that Chk2 is highly mobile before or after DNA damage, and immobilization of Chk2 to chromatin by fusing it with histone H2B attenuates its stimulating effect on p53-induced transcription, it has been concluded that the high mobility of Chk2 is essential for efficient signal transduction (29).

A simple model would be that, with free diffusion of Chk2 within the nucleus, a subpopulation that happens to be located near the sites of activated PIKKs would be phosphorylated and activated in turn. This is consistent with the finding that the chromatin-associated H2B-Chk2 fusion protein is indeed focally phosphorylated by laser-track irradiation (29). Similar to this report, we did not detect Thr68-phosphorylated Chk2 in the chromatin-enriched fraction after IR, Adriamycin, or camptothecin treatment. However, Chk2 was not recovered in a chromatin-enriched fraction in that study (29). The discrepancy may reflect differences in the experimental strategies or the different sensitivities of the anti-Chk2 antibodies. We have found that, although the majority of Chk2 molecules are recovered in the soluble fractions, a small pool of Chk2 is associated with the chromatin-enriched fraction. This population of chromatin-bound Chk2 is decreased after DNA damage. Previous studies using immunostaining of the bulk population of Chk2 were not able to detect the dynamic relocalization of this small pool of Chk2. Because of the small size of this pool, we cannot prove that this damaged subset is actually released from chromatin, rather than degraded. Nonetheless, the loss of Chk2 from the chromatin fraction, and the concomitant accumulation of phosphorylated Chk2 in soluble fractions, combined with the diminished partitioning of Chk2 phosphorylated in *vitro* to the chromatin pellet, are all consistent with an active release of Chk2 from chromatin once it has been activated. This conclusion is also consistent with the fact that irradiation-induced Chk2 dissociation from chromatin requires ATM, the major Chk2 upstream kinase, as well as the integrity of Chk2 SCD, FHA, and kinase domains that are required for damage-dependent Chk2 activation. Moreover, induced oligomerization of Chk2 combined with low level IR induced Chk2 release from chromatin.

The detection of Chk2 in the chromatin fraction in unperturbed cells suggests a role of Chk2 in normal cell cycles. This is reminiscent of the fact that some checkpoint signaling proteins execute non-checkpoint related functions to maintain genomic stability. For instance, in budding yeast, Rad53 regulates late replication origin firing (48), and is found to colocalize with replication foci in _S_ phase (49). Rad53 interacts with Dbf4, the regulatory subunit of Cdc7 kinase whose activity is required for the initiation of DNA replication (50). Rad53 interacts with Asf1, a chromatin assembly factor that acts in DNA replication and repair (51). Rad53 helps maintain telomere-proximal transcription repression as well as telomere length (52). In _Xenopus_ egg extracts, ATM and ATR regulate the timing of DNA replication origin firing in unperturbed cell cycles. Moreover, ATM is transiently activated during replication (53). A recent study also indicates the involvement of ATM, ATR, and Chk1 in the regulation of replication origins in mammalian cells (54). ATR associates with chromatin during _S_ phase (55). ATM-deficient cells show cell cycle-dependent telomere loss and end-to-end chromosome fusion, indicating a role of ATM in telomere maintenance (56). Chk1 binds to chromatin in cycling cells in the absence of DNA damage. UV-induced Ser^345^-phosphorylated Chk1 is predominantly associated with chromatin (57). Whether chromatin-associated Chk2 has another checkpoint-unrelated role warrants further investigation, including study of cell cycle regulation of Chk2 association with chromatin. A basal chromatin-associated fraction of Chk2 would be in an ideal position to receive the upstream DNA damage signal and relay the checkpoint response.

Our results, together with other published studies, support an overall model for Chk2 activation, in which a PIKK, recruited to chromatin after DNA damage, phosphorylates and activates a small pool of Chk2 that is associated with chromatin prior to damage, followed by the oligomerization of Chk2 molecules and release of hyperphosphorylated Chk2 from chromatin. Trans-autophosphorylation of oligomerized Chk2 in the soluble compartment permits the rapid transduction of damage signal and the prompt phosphorylation of downstream effector molecules.
