The protein kinase Chk2 has been implicated in signaling DNA damage to cell cycle checkpoints. In response to ionizing radiation, Chk2 becomes rapidly phosphorylated at threonine 68 by ataxia-telangiectasia mutated (ATM). Here we show that the Thr68-phosphorylated form of Chk2 forms distinct nuclear foci in response to ionizing radiation. Only this activated form of Chk2 localizes at sites of DNA strand breaks. The kinase activity of Chk2 and the number of Chk2 foci formed depend on the severity of DNA damage and gradually decline correlating with the predicted value of slowly rejoining double strand breaks. These results suggest that Chk2 is regulated at the sites of DNA strand breaks in response to ionizing radiation.

Chk2 (Cds1), an evolutionary conserved protein kinase, is an important component of the DNA damage response pathway. Chk2−/− ES cells are defective in maintaining ionizing radiation (IR)-induced G2 arrest, and Chk2 null thymocytes fail to stabilize p53 and to induce G1 arrest and apoptosis (1). Chk2 has also been reported to directly phosphorylate BRCA1 and to stabilize p53 and to induce G1 arrest and apoptosis (1). Chk2 is regulated at the sites of DNA strand breaks. These results suggest that Chk2 acts as a tumor suppressor.

Threonine 68 of Chk2 Is Phosphorylated at Sites of DNA Strand Breaks

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Irene M. Ward‡, Xianglin Wu‡, and Junjie Chen§
From the Division of Oncology Research, Mayo Clinic, Rochester, Minnesota 55905

The protein kinase Chk2 has been implicated in signaling DNA damage to cell cycle checkpoints. In response to ionizing radiation, Chk2 becomes rapidly phosphorylated at threonine 68 by ataxia-telangiectasia mutated (ATM). Here we show that the Thr68-phosphorylated form of Chk2 forms distinct nuclear foci in response to ionizing radiation. Only this activated form of Chk2 localizes at sites of DNA strand breaks. The kinase activity of Chk2 and the number of Chk2 foci formed depend on the severity of DNA damage and gradually decline correlating with the predicted value of slowly rejoining double strand breaks. These results suggest that Chk2 is regulated at the sites of DNA strand breaks in response to ionizing radiation.

The activation of Chk2 in response to DNA damage requires phosphorylation at threonine 68 (Thr68) (4–7). Chk2T68 mutants show reduced Chk2 kinase activation and a diminished induction of the p53-dependent G2 arrest in response to ionizing radiation (5, 6). The phosphorylation of Chk2 at Thr68 in response to IR is ATM (ataxia-telangiectasia-mutated)-dependent, although an ATM-independent pathway exists in response to ultraviolet radiation (UV) and hydroxyurea (HU) treatment (4–7). However, little is known how Chk2 activation is initiated or how the activity of Chk2 is down-regulated following DNA repair. Given the physiological impact of the DNA damage pathway in genome stability and cancer prevention, it is necessary to gain a better understanding of the mechanisms underlying DNA damage signal transduction. Here we show that the Thr68-phosphorylated form of Chk2 (Chk2T68P) forms distinct nuclear foci in response to ionizing radiation. Only this activated form localizes at sites of DNA strand breaks. The kinase activity of Chk2 and the number of Chk2 foci formed depend on the severity of DNA damage and gradually decrease with time. Together, our findings suggest that Chk2 activity is initiated and regulated at the sites of DNA strand breaks in response to ionizing radiation.

EXPERIMENTAL PROCEDURES

Constructs—The plasmids for the expression of HA-tagged wild-type and kinase-inactive Chk2 were kindly provided by Dr. J. Sarkaria, Mayo Clinic. The T68A mutation was constructed using the QuikChange site-directed mutagenesis protocol (Stratagene).

Antibodies—Rabbit and guinea pig polyclonal anti-Chk2T68P antibodies were raised against a peptide containing phospho-Thr68 (CETVST(PO4)QELYS). The antibodies were purified by affinity chromatography using the phospho-peptide linked to agarose beads (SulfoLink kit, Pierce) according to manufacturer’s instructions. Antibodies for Chk2 and GST fusion proteins containing full-length Chk2 (anti-Chk2 mAb#7) or the C-terminal residues 193–543 of Chk2 (anti-Chk2B). A mouse anti-53BP1 monoclonal antibody was raised against a mix of three GST fusion proteins encoding residues 1–337, 338–671, and 1331–1664 of 53BP1, respectively.

Immunoprecipitation and Western Blotting—Cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8), 0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride for 20 min on ice. Immunoprecipitation was carried out by incubating 4 μg of whole cell lysate with 1 μg of purified antibody and 20 μl of protein A-Sepharose beads (1:1) for 1 h at 4 °C. Beads were washed five times with NETN buffer. Bound proteins were eluted by boiling in SDS gel sample buffer, separated by SDS-PAGE, and transferred to Immobilon-P (Millipore). The polyvinylidene difluoride membrane was blocked with 5% nonfat milk for 30 min and incubated with 2 μg/ml anti-Chk2T68P or a 1:10 dilution of anti-Chk2 mAb#7 for 1 h. Horseradish peroxidase-conjugated goat anti-mouse (Amersham Pharmacia Biotech) or rabbit anti-mouse IgG were used as secondary antibodies. Immunoblotted proteins were visualized by chemiluminescence using the Supersignal kit purchased from Pierce.

In Vitro Kinase Assay—Immunoprecipitated Chk2 T68 was incubated with or without 1 μg of purified GST-Cdc25C (residues 200–256) for 30 min at 20 °C in 25 μl of kinase buffer (50 mM Tris (pH 7.5), 10 mM MgCl2, 10 μM ATP, and 15 μCi of [γ-32P]ATP. The reactions were stopped by the addition of 25 μl 2× Laemmli buffer. The samples were separated on a 12% SDS-PAGE, dried, and visualized by autoradiography.

Immunostaining—Cells grown on coverslips were fixed for 10 min in phosphate-buffered saline-buffered 3% paraformaldehyde, 2% sucrose solution and permeabilized for 5 min in ice-cold Triton buffer (0.5% Triton X-100 in 20 mM HEPES (pH 7.4), 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose). In case of anti-NBS1 staining, a 1:1 methanol:acetic acid solution was used to fix and permeabilize the cells. Immunostaining was performed using anti-Chk2 mAb#7 at a dilution of 1:10 or anti-Chk2T68P serum at a concentration of 0.35 μg/ml in 5% goat serum. Anti-53BP1 hybridoma supernatant (BP13) was used at a dilution of 1:30 and anti-NBS1 antibody (kindly provided by Dr. X. Wu, Dana Farber Cancer Institute) at a dilution of 1:5. The cells were incubated with the primary antibodies for 20 min at 37 °C, washed, and incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse and/or rhodamine-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratory, Inc.) at 1 to 200 dilution for 15 min. Cells were counterstained with Hoechst dye for 1 min. After mounting, the cells were viewed with a Nikon ECLIPSE E800 fluorescence microscope using a 60× objective, and images were processed using Adobe Photoshop and Canvas software.

Accelerated Publication
Phosphorylation and activation of Chk2 depend on the severity of DNA damage. To investigate the phosphorylation and activation of Chk2, we raised polyclonal anti-phospho-Thr68 antibodies against a Chk2 peptide containing T68P (CETTVST/PO4/QELYS). The specificity of these affinity-purified antibodies was examined by Western blot analysis. HA-tagged wild-type, a T68A mutant, or a kinase-inactive mutant (D347A) of Chk2 were transiently expressed in U2OS cells. Where the anti-Chk2 antibodies detected equal amounts of phosphorylated wild-type Chk2, but not the T68A mutant protein.

To further confirm that these antibodies only recognize phosphorylated Chk2 following DNA damage, we performed Western blot analysis using whole cell extracts prepared from 293T cells before and after exposure to 2–25 Gy of ionizing radiation. Whereas the anti-Chk2 antibodies detected equal amounts of total Chk2 in the various irradiated and non-irradiated samples, the anti-Chk2T68P antibodies recognized the phosphorylated Chk2 as a single ~70-kDa band in the irradiated samples (Fig. 1A, right panel). The intensity of this band increased with the radiation doses.

We then immunoprecipitated endogenous Chk2 from HeLa cell lysates prepared before and after treatments with HU, camptothecin (CPT), or IR. Whereas Chk2 protein levels did not change in response to various treatments, phosphorylation of Chk2T68 was only detected following exposure to IR or treatment with the topoisomerase I poison CPT, both of which induce DNA strand breaks (Fig. 1B, left panel). In agreement with the early finding that Thr68 phosphorylation is important for Chk2 activation (4–6), the kinase activities of Chk2, measured by autophosphorylation of Chk2 and phosphorylation of GST-Cdc25C (residues 200–256), increased following IR and CPT treatment (Fig. 1B, left panel). In contrast, only a slight increase in the phosphorylation of Chk2T68 was detected after a 1-h treatment with HU (Fig. 1B, left panel). Consistent with a role of ATM in the phosphorylation of Chk2, Thr68 phosphorylation and Chk2 activation were not detected in ATM-deficient FT169A cells after IR, while Chk2 was phosphorylated and activated in the isogenic, wild-type ATM-reconstituted YZ5 cells (Fig. 1B, right panel).

Increasing doses of γ-radiation resulted in increased levels of Chk2T68 phosphorylation (Fig. 1A, right panel; Fig. 1C, left panel) and a corresponding increase in Chk2 kinase activity (Fig. 1C, left panel). To examine the time course of Chk2 phosphorylation and activation, we irradiated HeLa cells with 5 Gy of IR and prepared lysates at various time points (0–160 min) after radiation. While the level of total Chk2 did not change over time, Chk2T68P increased in the first 20 min after radiation and then gradually decreased (Fig. 1C, right panel). A similar time course was observed for Chk2 kinase activities, as measured by Chk2 autophosphorylation and its ability to phosphorylate GST-Cdc25C (Fig. 1C, right panel). Taken together, these results suggest that the phosphorylation and activation of Chk2 correlate with the severity of DNA damage. Furthermore, dephosphorylation and inactivation of Chk2 correlate with the reported time course of DNA repair (8, 9).

Chk2T68P Forms Nuclear Foci in Response to IR—We next examined the subcellular distribution of Chk2 or Chk2T68P by indirect immunofluorescent analysis. The majority of Chk2 showed a diffuse nuclear staining pattern that did not appear to change upon exposure to IR (Fig. 2A). Whereas little or no Chk2T68P was detected in un-irradiated HeLa cells, distinct nuclear foci of Chk2T68P were observed following IR (Fig. 2A). The number of Chk2T68P foci increased in a dose-dependent fashion (Fig. 2A and data not shown) corresponding to the increase in Chk2T68 phosphorylation observed by Western analysis (Fig. 1C). Since the phosphorylation of Chk2 at Thr68 is predominantly dependent on ATM following IR (Refs. 4–7 and Fig. 1C), we examined Chk2T68P foci formation in wild-type versus ATM-deficient cells. While Chk2T68P foci formed readily in ATM+ YZ5 cells (Fig. 2B and data not shown), foci formation was greatly reduced in the isogenic ATM-deficient FT169A cells (Fig. 2B). In addition, pretreatment of HeLa cells with wortmannin, a fungal metabolite that inhibits phosphatidylinositol 3-OH-kinase-related kinases including ATM, blocked the formation of radiation-induced Chk2T68P foci (Fig. 2C).

Chk2T68P Co-localizes with 53BP1, γ-H2AX, and NBS1—The rapid, dose-dependent phosphorylation and activation of Chk2 in nuclear foci is reminiscent of the IR-induced phosphorylation of histone H2AX. Phosphorylated H2AX (γ-H2AX) forms distinct foci at sites of DNA strand breaks in response to DNA damage (10, 11). We (12) and others (13) have previously shown that 53BP1 rapidly co-localizes with γ-H2AX at the sites
of DNA strand breaks following IR. To examine whether phosphorylated Chk2 localizes at the sites of DNA strand breaks, we performed co-immunostaining experiments using anti-Chk2T68P and anti-53BP1 or anti-γ-H2AX antibodies. As shown in Fig. 3A, Chk2T68P foci overlapped with 53BP1 foci 1 h after exposure to 1 Gy of radiation. Similar staining patterns were also observed following CPT treatment (Fig. 3A), while short term treatment (1 h) with HU, which induced only a weak phosphorylation of Chk2T68 (Fig. 1B, left panel), did not lead to significant Chk2T68P foci formation (Fig. 3A). Co-immunostaining with anti-γ-H2AX confirmed that Chk2T68P foci localize at DNA strand breaks in response to IR (Fig. 3A and data not shown). As shown by earlier studies (10, 13) NBS1 co-localizes with γ-H2AX and 53BP1 several hours after IR. Likewise, NBS1 also co-localized with Chk2T68P in these late, IR-induced foci (Fig. 3C).

Time Course of Chk2T68 Foci Formation—Time course studies revealed that the phosphorylated form of Chk2 co-localized with 53BP1 within 10 min following IR and remained co-localized over the entire time interval tested (i.e. 10–160 min after IR; Fig. 4). Similar to γ-H2AX and 53BP1, the Chk2T68P foci number increased during the first 30 min and then gradually decreased thereafter. This decrease in the Chk2T68P foci number correlates with the decreases in the phosphorylation and kinase activity of Chk2 (Fig. 1C), suggesting that Chk2 may be dephosphorylated and inactivated at these DNA damage sites after DNA repair is accomplished.

**DISCUSSION**

Our data show that phosphorylation and activation of Chk2 by ATM correlate with the severity of DNA damage in vivo. The activated form of Chk2 localizes in distinct foci at the sites of DNA strand breaks within minutes following ionizing radiation. The disappearance of these foci coincides with that of γ-H2AX and 53BP1 and correlates with the reported time course of DNA repair (8, 9, 11, 12). The phosphorylation and activation of Chk2 by ATM occurs most likely at the sites of DNA breaks. First, ATM can be directly activated by DNA breaks in vitro (14, 15). Second, following DNA damage, ATM co-localizes with γ-H2AX foci in vitro (16). It is not yet known whether ATM directly phosphorylates Chk2 or this phosphorylation event requires a mediator. In budding yeast, phosphorylation and activation of the Chk2 homologue scRad53 by Mec1, an ATM homologue, require scRad9, a protein with C-terminal tandem BRCT motifs (BRCA1 C-terminus). It is intriguing that phosphorylated Chk2 co-localizes with 53BP1, a protein also containing C-terminal tandem BRCT motifs. It
remains to be determined whether the phosphorylation and activation of Chk2 requires 53BP1 in mammals.

The phosphorylation of Chk2 at Thr<sup>68</sup> does not correlate with the ATM-dependent mobility shift of Chk2 following ionizing radiation. While Chk2T68 phosphorylation is rapid and transient (see Fig. 1C), the mobility shift-associated hyperphosphorylation of Chk2 appears to be gradual and persists at least 24–48 h following DNA damage (17). Indeed, a Chk2T68A mutant, which cannot be phosphorylated at Thr<sup>68</sup> and is defective in activation following DNA damage, still shows the same mobility shift on SDS-PAGE as wild-type Chk2 (5). Thus, the mobility shift-associated hyperphosphorylation of Chk2 depends largely on the phosphorylation of sites distinct from Thr<sup>68</sup>. While Thr<sup>68</sup> phosphorylation of Chk2 correlates with the activation of Chk2 (4–7), the function of this mobility shift-associated hyperphosphorylation of Chk2 remains to be solved. One possibility is that this mobility shift-associated hyperphosphorylation of Chk2 represents a certain negative feedback mechanism. Such complex regulation of kinase activity by various phosphorylation events has been documented for Raf-1 protein kinase. While Raf-1 is initially activated by specific phosphorylation events, the subsequent mobility shift-associated hyperphosphorylation of Raf-1 down-regulates Raf-1 activity and represents a negative feedback mechanism contributing to the desensitization of the signaling pathway (for example, see Ref. 18). Similar feedback mechanism may exist for Chk2 in the DNA damage-signaling pathway.

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**REFERENCES**

1. Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000) *Science* **287**, 1824–1827
2. Lee, J. S., Collins, K. M., Brown, A. L., Lee, C. H., and Chung, J. H. (2000) *Nature* **404**, 201–204
3. Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wuhrer, D. C., Shannon, K. E., Lubratiovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., Birch, J. M., Li, F. P., Garber, J. E., and Haber, D. A. (1999) *Science* **286**, 2528–2531
4. Ahn, J. Y., Schwarz, J. R., Piwnica-Worms, H., and Canman, C. E. (2000) *Cancer Res.* **60**, 5934–5936
5. Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 10389–10394
6. Melchionna, R., Chen, X. B., Blasina, A., and McGowan, C. H. (2000) *Nat. Cell Biol.* **3**, 762–765
7. Zhou, B. B., Chataturvedi, P., Spring, K., Scott, S. P., Johanson, R. A., Mishra, R., Mattern, M. R., Winkler, J. D., and Khanna, K. K. (2000) *J. Biol. Chem.* **275**, 10342–10348
8. Kodym, R., and Horith, E. (1995) *Int. J. Radiat. Biol.* **68**, 133–139
9. Nunez, M. I., Villalobos, M., Olea, N., Valenzuela, M. T., Pedraza, V., McMillan, T. J., and Ruiz de Almodovar, J. M. (1995) *Br. J. Cancer* **71**, 311–316
10. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M. (2000) *Curr. Biol.* **10**, 886–895
11. Rogakou, E. P., Been, C., Redon, C., and Bonner, W. M. (1999) *J. Cell Biol.* **146**, 905–916
12. Rappold, I., Iwabuchi, K., Date, T., and Chen, J. (2001) *J. Cell Biol.* **153**, 613–620
13. Schultz, L. B., Chehab, N. H., Malikzay, A., and Halazonetis, T. D. (2000) *J. Cell Biol.* **151**, 1381–1390
14. Smith, G. C., Cary, R. B., Lakin, N. D., Hannon, B. C., Tro, S. H., Chen, J. D., and Jackson, S. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 11134–11139
15. Chan, D. W., Sun, S. C., Block, W., Ye, R., Khanna, K. K., Wold, M. S., Douglas, P., Goodarzi, A. A., Pelley, J., Taya, Y., Lavin, M. F., and Lees-Miller, S. P. (2000) *J. Biol. Chem.* **275**, 7803–7810
16. Andegeko, Y., Moyal, L., Mitelman, L., Tsarfaty, I., Shiloh, Y., and Rotman, G. (2001) *J. Biol. Chem.* **276**, 38224–38230
17. Buscemi, G., Savio, C., Zannini, L., Muccio, F., Masnada, D., Nakashiki, M., Tsoukis, H., Komatsu, K., Mizutani, S., Khanna, K., Chen, P., Concannon, P., Chessa, L., and Delia, D. (2001) *Mol. Cell. Biol.* **21**, 5214–5222
18. Wartmann, M., Hofer, P., Turowski, P., Saltiel, A. R., and Hynes, N. E. (1997) *J. Biol. Chem.* **272**, 3915–3923