4 Chk2 and the centrosomal response to genotoxic stress.

Henderika M.J. Hut, Krzysztof P. Rembacz, Harm H. Kampinga, and Ody C.M. Sibon

Centrosomes are target organelles that are affected when cells enter mitosis in the presence of impaired DNA integrity. In Drosophila embryos Chk2 is localized at centrosomes and plays an essential role in activating the centrosome response pathway after DNA damage (18). In untreated mammalian cells Thr68-phosphorylated Chk2 is present at centrosomes (19) suggesting a possible role of mammalian Chk2 in centrosome function and centrosome responses as well. Here we investigate and discuss the centrosomal role of mammalian Chk2 when irradiated cells enter mitosis in the presence of damaged DNA.
Introduction

Centrosomes play an essential role in microtubule organization and are required for proper segregation of replicated chromosomes during mitosis (reviewed in (9)). Recently, it has been demonstrated that centrosomes are also involved in a variety of cellular stress responses (5-7; 17; 18). In Drosophila embryos, genotoxic stress triggers loss of specific centrosomal proteins during mitosis. This leads to centrosome disruption, defects in spindle assembly and this prevents anaphase chromosome segregation. The damaged nuclei are eliminated from the embryonic precursor pool leading to normal development of the embryo proper (17; 18). In mammalian cells, centrosomes also respond to the presence of impaired DNA integrity during mitosis (6). However, in contrast to centrosome inactivation in Drosophila embryos, mammalian centrosomes split into monocentriolar fragments forming multipolar spindles in the presence of incompletely replicated DNA. When cells with multipolar spindles progress through mitosis a variety of division errors can occur, such as the formation of non-equal daughter cells, the formation of three or more daughter cells or the formation of binuclear cells (6). In response to irradiation, mammalian centrosomes do not split during mitosis, however, centrosome function may be affected since cytokinesis failure was frequently observed after irradiation (7). It is yet unclear whether division errors as observed in mammalian cells in response to the presence of impaired DNA integrity during mitosis lead to elimination of the cells. Summarizing, although centrosomes in Drosophila and mammalian cells respond differently, centrosomes seem to be target organelles that are affected in the presence of impaired DNA integrity during mitosis in both organisms.

In Drosophila embryos the Checkpoint kinase 2 (DmChk2/Dmnk) is essential in the centrosomal inactivation response to DNA lesions as in DmChk2/Dmnk null mutants this mitotic response is blocked and defective nuclei remain present at the cortex (18). In mammalian cells, Chk2 is an effector kinase playing a role in the DNA damage response pathway (reviewed in (15)). In untreated cells Chk2 is not phosphorylated and upon irradiation it becomes phosphorylated through ATM-dependent phosphorylation at threonine 68 (12). This Thr68-phosphorylation is essential for the initial oligomerization (1), autophosphorylation on Thr383 and Thr387 and kinase activation of Chk2 (16). Activated Chk2 can phosphorylate p53 (2) and Brca1 (10) and remarkably cells with mutations in either p53 or Brca1 were found to have abnormal numbers of centrosomes (4; 21) leaving a potential role for their upstream protein kinase Chk2 in a centrosomal response to DNA damage. In addition, by the use of immunofluorescence it has been demonstrated that Thr68-phosphorylated Chk2 localizes at centrosomes (19). This suggests that Chk2 might be involved in centrosomal reactions during the DNA damage response pathway via its Thr68 phosphorylation status. Given that in Drosophila embryos the DmChk2/Dmnk-dependent centrosomal inactivation response was only observed during mitosis (18), a potential mammalian Chk2-dependent centrosomal response to DNA damage might also occur exclusively during the mitotic phase.

Here, we tested whether there is a direct link between Thr68-phosphorylated Chk2 and centrosome changes in response to the presence of damaged DNA in mitosis. We demonstrate that antibodies directed against Thr68-phosphorylated Chk2 bind non-specific to centrosomes in immunofluorescence experiments and therefore a direct interaction of Thr68-phosphorylated Chk2 with DNA damaged centrosomes cannot be verified nor excluded based on these experiments. Based on our findings and recent findings by other groups a possible involvement of Chk2 in a mammalian centrosome response pathway is being discussed.

Results + Discussion

In order to investigate a possible role of Thr68-phosphorylated Chk2 in centrosomal responses to impaired DNA integrity, we first tested the specificity of the commercially available Thr68-Chk2 antibody against Thr68-phosphorylated Chk2 by the use of Western blotting. In untreated HeLa cells, the Chk2 antibody showed one specific band at 62 kDa
representing the non-phosphorylated Chk2 protein (Figure 1A, lane 1). This signal did not significantly change after irradiation and recovery (Figure 1A, lane 2 and 3). As expected, the Thr68-Chk2 antibody showed only a weak signal in untreated cells (Figure 1B, lane 1) and did reveal a strong specific band in irradiated cells representing Thr68-phosphorylated Chk2 (Figure 1B, lane 2 and 3). These results are in agreement with previous studies demonstrating that Chk2 is phosphorylated at Thr68 after irradiation (12; 13; 20) and reveal that the used antibody specifically binds to Thr68-phosphorylated Chk2 on Western blots.

**Figure 1. Antibodies against Chk2 and T68-phosphorylated Chk2 are specific on Western blot.**

Lysates of HeLa cells expressing centrin-GFP, untreated (lane 1) or irradiated with 5 Gy followed by recovery of 3 (lane 2) or 6 hours (lane 3), were equally loaded on a polyacrylamide gel and expression levels of Chk2 (A) and Thr68-phosphorylated Chk2 (B) were detected. The anti-Chk2 antibody labels a strong band in all samples and a faint band, representing phosphorylated Chk2, with lower mobility after irradiation. The anti-Thr68-Chk2 antibody labels a faint band in untreated cells and labels a strong band after irradiation, representing Thr68-phosphorylated Chk2.

Immunofluorescence was used to visualize Thr68-phosphorylated Chk2 localization in control cells and in irradiated cells. For this purpose, mitotic HeLa cells were used that stably express the centrosomal protein centrin fused to GFP (14). In untreated mitotic cells the Thr68-Chk2 antibody colocalized with centrin-GFP at centrosomes (Figure 2A-D). The Thr68-Chk2 antibody co-localized with interphase centrosomes as well (data not shown). Centrosomal staining of the Thr68-Chk2 antibody is in accordance with previous published results in HEK-293T, WI38, HT-1080 and U2OS cells (19). Remarkably, in our experiments the Thr68-Chk2 antibody also localized at the spindle microtubules in a punctated pattern (Figure 2A and I arrows), which has not been described before. One explanation may be that Thr68-Chk2 localization at microtubule filaments is specific for HeLa cells. After irradiation the centrosomal staining of the Thr68-Chk2 antibody at centrosomes remained unaltered (Figure 2E-H). In contrast, the foci located at microtubule filaments were not visible after irradiation (Figure 2E), which suggests that Thr68-phosphorylated Chk2 disappears from the spindle microtubules in response to irradiation.

The strong Thr68-Chk2 antibody staining of centrosomes and mitotic spindles in control cells, as visualized by immunofluorescence (Figure 2A-D) is not in accordance with the weak Thr68-Chk2 band observed in control cells as visualized by Western blotting (Figure 1B, lane 1). It is possible that the Thr68-Chk2 antibody recognizes Thr68-phosphorylated Chk2 more efficiently by immunofluorescence (proteins are fixed with methanol/ acetone) than by Western blotting (denatured proteins). It is also possible that the Thr68-Chk2 antibody staining observed after immunofluorescence is not specific. To examine the specificity of the Thr68-Chk2 antibody for immunofluorescence, we used the HCT-15 cell line that is impaired in the ATM-dependent phosphorylation of the Thr68-site of Chk2 (3). Experiments on Western blot showed that the Thr68-Chk2 antibody indeed showed a very weak signal in untreated as well as in irradiated HCT-15 cells (Figure 3, lane 4-6) whereas HeLa cells did reveal a strong specific band only after irradiation (Figure 3, lane 2 and 3). However, when the immunofluorescence experiments as demonstrated in Figure 2 were repeated using HCT-15 cells, labeling of the Thr68-Chk2 antibody at centrosomes and microtubules was
observed similar as in HeLa cells (Figure 4). These results demonstrate that the Thr68-Chk2 antibody specifically recognizes Thr68-phosphorylated Chk2 on Western blots, but that this antibody non-specifically binds to (a) protein(s) other than Thr68-phosphorylated Chk2 in immunofluorescence experiments. It is yet unclear which centrosome and spindle protein(s) are recognized by the Thr68-Chk2 antibody. As far as we know, a protein that is localized in a punctated pattern along microtubules and that translocates after irradiation has not been described previously. It will be of interest to identify this protein and to investigate its possible role in DNA damage response pathways.

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**Figure 2. Anti-Thr68-Chk2 antibodies label spindle microtubules and centrosomes in untreated mitotic cell and after irradiation anti-Thr68-Chk2 antibodies disappear from the spindle whereas centrosomal labeling remains unaltered.**

HeLa cells stably expressing centrin-GFP (green), left untreated (A-D, I) or 5 Gy irradiated followed by 4 hours recovery (E-H), were fixed and labeled with anti-Thr68-Chk2 antibodies (red). DNA was stained by DAPI (blue). A, E, I Thr68-Chk2; B, F, centrin-GFP; C, G, DAPI; D, H, overlay. Bar, 10 μm. Arrows mark labeling of anti-Thr68-Chk2 antibodies at microtubules during mitosis.

Our data revealed a nonspecific centrosome and spindle staining of the Thr68-Chk2 antibody against Thr68-phosphorylated Chk2. Previously, it has been demonstrated that other antibodies raised against Thr68-phosphorylated Chk2 also aspecifically decorate nuclear foci after irradiation (11). These results and our results indicate that data obtained from immunofluorescence experiments using these Thr68-Chk2 antibodies are not reliable and conclusions regarding the involvement of Chk2 in a centrosome damage response pathway cannot be drawn.
Chk2 and the centrosomal response to genotoxic stress

Figure 3. Western blot analysis demonstrates that phosphorylation of Chk2 does not occur in HCT-15 cells after irradiation.

HeLa centrin-GFP cells (lane 1,2,3) and HCT-15 cells (lane 4,5,6), left untreated (lane 1, 4) or irradiated with 5 Gy followed by recovery of 5 minutes (lanes 2,5) or 4 hours (lanes 3,6), were prepared for Western blot analysis and probed with anti-Thr68-Chk2 antibody. Anti-γ-tubulin was used as loading control. Thr68-phosphorylation of Chk2 was only observed in irradiated HeLa cells and not in HCT-15 cells.

In contrast to Thr68-phosphorylated Chk2, a specific co-localization of Thr387-phosphorylated Chk2 was observed three hours after ionizing irradiation in interphase cells using immunofluorescence (8). The reliability of this signal was demonstrated by the fact that down regulation of endogenous Chk2 by RNAi led to an almost complete disappearance of centrosomal signal after irradiation (8). Krämer et al did not report data concerning centrosome localization of Thr387-phosphorylated Chk2 on irradiated cells during mitosis nor did they verify the immunofluorescence data by localization studies using Chk2-GFP fusion proteins. Remarkably, in an earlier study this research group did perform studies using Chk2-GFP fusion proteins, however in that report a centrosomal localization of Chk2-GFP was not mentioned and only the nuclear localization pattern of Chk2-GFP in control cells and after irradiation was described and discussed (11). Based on the results as we describe here and based on our previous studies (7) data concerning centrosomal localization of proteins based on antibodies and immunofluorescence need to be verified using mutant cell lines, RNAi technology and GFP fusion proteins. Therefore, currently it is not possible to conclude anything about a possible role of (phosphorylated) Chk2 in centrosomal responses to DNA damage in mammalian cells.

Chk1, another effector kinase playing a role in DNA damage responses was shown to localize at centrosomes by immunofluorescence and this localization was verified using Chk1-GFP fusion proteins (8). RNAi treatment resulted in an almost complete disappearance of centrosomal staining of Chk1 antibodies, indicating that Chk1 indeed is localized at centrosomes (8). Kramer et al furthermore showed that Chk1 does localize to centrosomes during interphase, but not during mitosis and their results point towards a role of centrosome-associated Chk1 in proper timing of the initial steps of cell division. Now it remains to be investigated whether centrosomal Chk1 localization is affected after irradiation and whether Chk1 does play a role in the centrosomal DNA damage response pathway in mammalian cells. In Drosophila embryos it has been shown that Chk2 but not Chk1 is involved in the centrosomal inactivation pathway during mitosis (17; 18). In mammalian cells Chk1 is (and most likely Chk2 is not) localized at centrosomes, suggesting that, in contrast to Drosophila cells, in mammalian cells Chk1 and not Chk2 is playing a role in centrosomal responses to impaired DNA integrity and during evolution from flies to mammals a functional switch may have occurred between Chk1 and Chk2.
Chapter 4

Figure 4. Labeling of anti-Thr68-Chk2 is similar in HeLa cells and in HCT-15 cells deficient in Chk2 phosphorylation.

HCT-15 cells, left untreated (A-C) or 5 Gy irradiated followed by 4 hours recovery (D-F), were fixed and labeled with anti-Thr68-Chk2. DNA was stained by DAPI. A, D, Thr68-Chk2 - CY3; B, E, DAPI; C, F, overlay. Bar, 10 µm.

Materials and Methods

Cell Lines and Culturing
HeLa centrin-GFP cells (kindly provided by M. Bornens (14)) were cultured in DMEM (high glucose) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 600 µg/ml genetin (GIBCO, Paisley, UK). HCT-15 cells (CCL-225, American Type Culture Collection, Manassas, USA) were cultured in RPMI 1640 supplemented with 10% FBS. All cultures were maintained at 5% CO₂ in a humidified 37°C incubator.

Treatment
Ionizing radiation was applied using radiation from a ¹³⁷Cs source in an IBL 637 irradiator (CIS Biointernational, France). After 5 Gy irradiation, cells recovered for indicated times in a humidified 37°C incubator.

Immunofluorescence Analysis
For immunofluorescence analysis cells were grown on coverslips and fixed using methanol/acetone as previously described (6). To visualize the localization of Thr68-phosphorylated Chk2, polyclonal anti-P-Chk2 (sc-16297-R, Santa Cruz Biotechnology, Santa Cruz, California) was used in combination with anti-rabbit CY3 (Amersham, Piscataway, NJ). To visualize the DNA, cells were stained for 10 min with 0.2 µg/ml DAPI. Images were obtained with a confocal laser-scanning microscope (Leica TCS SP2, Leica Microsystems, Heidelberg, Germany) with 351/364-, 488-, and 543-nm lasers.

Western Blot Analysis
Proteins were separated by SDS/ 10% polyacrylamide gels and transferred to nitrocellulose membranes. Chk2 and Thr68-phosphorylated Chk2 were detected with polyclonal anti-Chk2 (Chk2 (H-300), sc-9064, Santa Cruz) respectively polyclonal anti-P-Chk2 (sc-16297-R, Santa Cruz) primary antibodies. As secondary antibody HRP-conjugated goat-anti-rabbit was used (Amersham). Signals were detected by enhanced chemiluminescence (ECL, Amersham).
Chk2 and the centrosomal response to genotoxic stress

References


