Heat-Induced Perturbations of DNA Damage Signaling Pathways are Modulated by Molecular Chaperones

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Abstract

Heat is one of the most potent radiosensitizers known. Several randomized trials have shown that hyperthermia is a good adjuvant for radiotherapy at several different cancer sites. However, the mechanism(s) involved in the interaction of heat and radiation that lead to radiosensitization remain to be elucidated. In this report, we have determined that heat induces perturbations in some of the earliest events in the cellular response to DNA damage induced by ionizing radiation. We studied the effect of heat on the formation of complexes containing γ-H2AX/MDC1/53BP1 in heated-irradiated cells. We found that the formation of this complex was delayed in heated-irradiated cells, in a heat but not radiation dose–dependent manner. The length of the heat-induced delay of complex formation was attenuated in thermotolerant and heat radiosensitization–resistant cells. The length of the delay of γ-H2AX/MDC1/53BP1 complex formation correlated with the magnitude of heat radiosensitization and was modulated by the molecular chaperone Hsc70. Heat radiosensitization was attenuated in 53BP1-null cells, implying that the delay of the formation of the γ-H2AX/MDC1/53BP1 complex plays a role in heat radiosensitization. Heat also induced a delay of events in the DNA damage response that are downstream from 53BP1. Our results support the notion that heat-induced perturbations in the earliest events of the cellular response to ionizing radiation–induced DNA damage play a role in heat radiosensitization. [Cancer Res 2009;69(5):2042–9]

Introduction

Heat is a very potent sensitizer to the action of ionizing radiation (IR), both in cells in culture and in human tumors (1, 2). Recent randomized phase III clinical trials have indicated that the combination of conventional radiation therapy with hyperthermia leads to significantly better tumor control of malignant melanomas, recurrent breast carcinomas, deep pelvic tumors, and glioblastomas (2). Understanding the mechanism of action of this heat radiosensitizer would yield information with translational implications, both in terms of improving the efficacy of the combined modality in the clinic, and the possible development of agents that mimic the radiosensitizing action of heat. Exposure to heat leads to alterations in chromatin structure, which reduce the accessibility to the DNA repair machinery, leading to the perturbations of DNA double-strand break (DSB) repair associated with heat sensitization (1, 3–5). Heat also induces cellular signaling pathways and perturbations in signaling pathways induced by other agents (6). Thus, heat radiosensitization may also involve heat-induced alterations/perturbations of the signaling pathways associated with the cellular response to IR-induced DNA damage (7). This is the hypothesis that we tested in this report.

Recently, the cellular response to DNA DSB has been conceived as a combination of classic signal transduction cascades triggered by a series of phosphorylation-dependent events in which a "signal" (DNA damage) is detected by "sensors" (DNA-damage binding proteins), which then triggers the activation of a transducing system (protein kinase cascade), which in turn amplifies and diversifies the signal targeting of a series of downstream "effectors" of the DNA damage response (7). In this model, the nonhomologous end joining (NHEJ) and the homologous recombination (HR) repair pathways for DNA DSBs are viewed as effectors (7). Heat radiosensitization involves impairment of the repair of radiation-induced DNA DSB (1, 4, 5). Because the magnitude of heat radiosensitization is not diminished in cells that are mutated in, or have deletions of, several different key components of the NHEJ and HR repair pathways, these pathways are not direct targets of heat radiosensitization (5). Given these results, the target(s) of heat radiosensitization may be upstream of the effectors of the cellular response to DNA DSB. Thus, proteins that perform sensor or "transducing/mediating" functions are likely to be targets for heat radiosensitization.

Several of the "sensor" and transducing proteins become rapidly associated with the region around newly generated DNA DSB. Within <1 min after irradiation, cytologically observable foci, the IR-induced foci (IRIF; refs. 8, 9), containing several sensors, DNA DSB, including the Mre11/Rad 50/Nbs1 (MRN) complex, γ-H2AX, MDC1, BRCA1, and p53-binding protein 1 (53BP1; refs. 8, 9). One of the earliest IRIF, containing γ-H2AX, the phosphorylated form of histone H2AX, has been suggested to play a direct role in the organization of foci containing proteins involved in the repair of DNA DSB (8–10).

Heat has been reported to induce γ-H2AX foci (11–13). The former two reports have postulated that such foci represent heat-induced DNA DSB that play a role in heat-induced cell killing. In a recent report, we found that whereas heat induces γ-H2AX foci (60–70 γ-H2AX foci after 60 min at 43 °C, the number of foci that is found after 2 Gy of IR), it does not induce DNA DSB after this heat dose, as measured by three different techniques. The limit of the resolution of one of our techniques, G2 chromosomal aberrations, was ~4 to 9 DNA DSB (13). Furthermore, the induction of γ-H2AX foci was not accompanied by the formation of foci containing several other proteins that are involved in the repair of DNA DSB, including 53BP1 (13). In addition to the induction of γ-H2AX containing foci, heat also induces foci containing MDC1 (13). Foci containing both of these proteins are a prerequisite for the formation of foci containing 53BP1 (10, 14), which were not observed after heat (13). 53BP1 forms IRIF, thought to represent...
sites of DNA DSBs because they colocalize with other IRIF known to mark sites of DNA DSBs such as γ-H2AX, MDC1, and the MRN complex (9, 14). Therefore, we decided to determine the effect of heat on the formation of IRIF containing 53BP1 in heated-irradiated cells as a test of the hypothesis that heat-induced perturbations in the early response to radiation-induced DNA DSB play a role in heat radiosensitization.

We envision that a complex containing γ-H2AX, MDC1, and 53BP1 is formed in irradiated cells based on the following observations. The IRIF containing these three proteins colocalize as indicated by high-resolution immunofluorescence; the induction and resolution of such IRIF occur in parallel (8, 14). The formation of γ-H2AX and MDC1 IRIF is interdependent because no MDC1 IRIF are found in H2AX−/− mouse embryonic fibroblasts (MEF; ref. 10) and few γ-H2AX foci are found in MDC1−/− MEF (8). Both γ-H2AX and MDC1 foci are found in 53BP1−/− MEF (15). On the other hand, no 53BP1 IRIF are found in H2AX−/− or MDC1−/− MEFs (8, 10). Thus, the presence of a complex of γ-H2AX and MDC1 is necessary for the formation of the γ-H2AX/MDC1/53BP1 complex (for the sake of brevity, we shall refer to the γ-H2AX/ MDC1/53BP1 complex as the "53BP1 complex"). Knocking out any component of this complex leads to radiosensitivity (8, 14).

In this report, we found that heating prior to or after irradiation delays the formation of 53BP1-containing foci, thus indicating that heat interferes with the formation of the 53BP1 complex. We characterized the heat-induced delay in the formation of the 53BP1 complex in heated-irradiated cells, the factors that attenuate or enhance it, and present evidence that 53BP1 plays a role in heat radiosensitization.

**Materials and Methods**

**Cell lines and growth conditions.** Confluent or serum-starved cells were used throughout these studies to avoid cell cycle effects. Parental wild-type HA-1 and heat radiosensitization–resistant HR-1 cells were grown as described (16). MCF7, HeLa cells, HEK293, and 53BP−/− and 53BP−/− MEFs were grown in DMEM containing 10% FCS and antibiotics, with the addition of pyruvate and β-mercaptoethanol for the growth of the MEFs (15).

**Radiation treatments.** Control cells, preheated cells, and cells which were heated after irradiation were all irradiated using a Pantak model PMC1000 X-ray generator, operated at 250 kV and 12 mA. Irradiation was performed in a chamber containing 5% CO2 at 37°C. For cells exposed to combined treatments, the time interval between the various treatments was <2 min.

**Heating and thermodreration induction.** Tissue culture dishes were sealed with parafilm, kept in a 37°C water bath for 10 min and then inserted into a precision-controlled (±0.05°C) water bath for appropriate lengths of time. Transient thermodreration was induced as described (17, 18). Cells were heated at 43°C for 30 min, allowed to recover at 37°C for 24 h, and then challenged by a treatment of 60 min at 43°C.

**Immunofluorescence.** For immunofluorescence studies, cells were grown for 48 h before experiments on acid-etched coverslips as described (18). After various experimental treatments, the cells were fixed and processed for immunofluorescence as described (18). Briefly, cells were washed, and then fixed with 3.7% formaldehyde in PBS containing 0.2% Triton X-100, then followed by a 10 min extraction with acetone at −20°C. Alternative fixation protocols gave essentially the same results. Antibodies against γ-H2AX were obtained from Upstate Biotech, an anti-53BP1 monoclonal antibody and a polyclonal antibody against MDC1 were a gift from Dr. J. Chen (Department of Radiation Oncology, Yale University, New Haven, CT), a polyclonal anti-S3BP1 antibody was obtained from Novus, Anti-p95/S MCM1 and anti-Chk2 p68T antibodies were obtained from Cell Signaling. Secondary antibodies, labeled with Alexa Fluor, were obtained from Molecular Probes. Images were captured using a 100×/Plan Apo NA1.4 objective of an Olympus BX 40 microscope and a Diagnostic Instruments Spot Pursuit CCD camera.

For hamster cells, cells containing less than four 53BP1 foci were counted as negative and cells containing more than four foci were counted as positive, whereas for MCF7 and HeLa cells, we used six foci as a cutoff point. Similar protocols have been used by several investigators who have quantified the formation and resolution of various IRIF (19–21).

**Extraction of 53BP1 monitored by quantitative immunofluorescence.** Extraction and determination of the solubility of 53BP1 by immunofluorescence were performed as described (22). Briefly, after various experimental treatments, cells were extracted in situ for 20 min at 4°C in cytoskeletal buffer [50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 3 mmol/L MgCl2, 300 mmol/L sucrose, 0.5% NP40, containing protease and phosphatase inhibitors]; control cells were treated with the same buffer lacking NP40. Cells were then fixed and stained with the appropriate antibodies. For quantitative analysis, the intensity of 50 random cells in images obtained with the same exposure time was quantitated using Image Pro 6.

**Immunoblotting.** Immunoblotting and fraction cell preparation was performed as described (18), except that the alkaline phosphatase detection system was used.

**Hsc70 constructs.** Wild-type Hsc70 and dominant negative D199S Hsc70 containing adenosine constructs were a gift from Drs. S. Schmidt and S. Newmyer (Department of Cell Biology, Scripps Research Institute, La Jolla, CA), and were generated as described (23). The viruses were grown in HEK293 cells and purified by CsCl density gradient sedimentation. Exponentially growing HeLa cells were infected at a multiplicity of infection of 200 by a 2-h incubation at 37°C with the viruses, and then allowed to recover for 16 h at 37°C before exposure to various experimental treatments.

**Clonogenic survival curves.** Clonogenic survival curves were generated as described (16). We did not find any trypsin effects for radiation or the combination of heat and irradiation (16).

**Results**

**Heating delays the formation of the 53BP1 complex induced by radiation.** Heat treatment alone induced γ-H2AX and MDC1 foci but not 53BP1 foci (13), suggesting that the 53BP1 complex did not form in heated cells. Thus, we could study the effect of heat on the formation of the 53BP1 complex induced by radiation. Heat treatment of irradiated cells delayed the formation of the 53BP1 complex by 4 to 6 hours following combined treatments, regardless of the order of irradiation and heating (Fig. 1A–C). Such delay was observed in heated-irradiated hamster, mouse, and human cells, normal and transformed, and independent of p53 status. The delay in 53BP1 complex formation in heated-irradiated cells was observed with five different anti-53BP1 antibodies, indicating that such delay was not due to heat-induced epitope masking (data not shown). The heat-induced delay in 35BP1 complex formation was reversible, as indicated by irradiating at various times post-heating (Supplementary Fig. S1). The delay in the formation of the 53BP1 complex was induced by as little as 15 minutes at 43°C in a heat dose–dependent manner, but was radiation dose–independent, as indicated by dose-response curves, although there was a radiation dose–dependent increase in the number of 53BP1 foci per cell (Fig. 2A and B). Although the observation that even after 12 Gy only 80% to 85% of cells displayed 53BP1 IRIF is different than that reported previously (22), we obtained similar results with several different human cancer cell lines (data not shown). The recovery from the delay of 53BP1 complex formation in heat-irradiated cells was independent of de novo protein synthesis, indicating a lack of rapid turnover of 53BP1 during the delay (Supplementary Fig. S2).

Furthermore, we found that heating at various times post-irradiation led to the “dispersal” of previously formed radiation-induced 53BP1 complexes (Supplementary Fig. S3).

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induced delay in 53BP1 complex formation (Fig. 1A–C). Similar results were obtained when the insoluble pellet from cell fractionation experiments (22) was analyzed by immunoblotting (Supplementary Fig. S4). Thus, the lack of association of 53BP1 with γ-H2AX/MDC1 complexes in heated and heated-irradiated cells was not associated with a global heat-induced aggregation and/or insolubilization of 53BP1.

**Molecular chaperones modulate the heat-induced delay of 53BP1 complex formation.** To ascertain if molecular chaperones can modulate the heat-associated delay in 53BP1 complex formation, we determined the response of transiently thermotolerant (TT), cells. Thermotolerance is the ability of cells which have been exposed to nonlethal heat doses to display increased survival after treatment with otherwise lethal heat doses, and is associated with elevated expression of several molecular chaperones (3, 17, 24). Heat radiosensitization was attenuated in thermotolerant cells (Supplementary Fig. S5), as measured by the thermal enhancement ratio (TER; Supplementary Table S1), which is the ratio of the radiation dose alone to that in combination with heat to achieve isosurvival (1). The initial magnitude and the decay of the heat-induced delay in 53BP1 complex formation was attenuated in

![Figure 1](https://example.com/figure1.jpg)

**Figure 1.** Delay of 53BP1 complex formation in heated-irradiated cells. A, MCF7 cells were heated for 60 min at 43°C and then irradiated with 2 Gy and stained for 53BP1 foci at various times posttreatment. IRc, 2 Gy followed by a 30-min recovery; HR, cells heated and then irradiated. B, MCF7 cells were irradiated with 2 Gy and then heated for 60 min at 43°C and stained for 53BP1 foci at various times posttreatment. Rc, 2 Gy-30 min recovery; RH, cells irradiated and then heated. C, time course of the heat-induced delay of 53BP1 complex formation in heated then irradiated (●) and irradiated then heated MCF7 (▲) cells. Control irradiated (■) cells. The experimental conditions were identical to those described for A and B. After various times posttreatment, the fraction of cells containing more than six 53BP1 foci was determined.

Because transient heat-induced insolubilization and/or aggregation of 53BP1 would prevent it from being associated with the γ-H2AX/MDC1 complex in heated-irradiated cells, we determined if heat altered the solubility of 53BP1 by performing in situ cell extractions with a buffer containing a nonionic detergent, followed by quantitative immunofluorescence (Fig. 3A and B). We obtained the following results in this experiment. In control cells, ~50% of 53BP1 was insoluble; there is an increase in the insolubility of 53BP1 in irradiated cells, confirming the results of others (22). On the other hand, heat prevented the radiation-induced insolubilization of 53BP1 (Fig. 3B). This result was consistent with the heat-induced delay in 53BP1 complex formation (Fig. 1A–C). Similar results were obtained when the insoluble pellet from cell fractionation experiments (22) was analyzed by immunoblotting (Supplementary Fig. S4). Thus, the lack of association of 53BP1 with γ-H2AX/MDC1 complexes in heated and heated-irradiated cells was not associated with a global heat-induced aggregation and/or insolubilization of 53BP1.

![Figure 2](https://example.com/figure2.jpg)

**Figure 2.** Dose-response of the delay in 53BP1 complex formation in heated-irradiated cells. A, MCF7 cells were irradiated with 2 Gy followed by heating at 43°C for various lengths of time. 2 Gy alone (●), 2 Gy followed by treatment at 43°C for 15 min (▲), 30 min (◆), or 60 min (▼); the fraction of cells containing more than six 53BP1 foci was determined at various times posttreatment. B, MCF7 cells were irradiated with 4 Gy (●), 8 Gy (◆), or 12 Gy (▲) of X-rays, followed by heating at 43°C for 60 min and the fraction of cells containing more than six 53BP1 foci was determined at various times posttreatment.
thermotolerant cells (Fig. 4A), indicating that in thermotolerant cells, the attenuation of the heat-induced delay of 53BP1 complex formation is associated with a decrease in TER. Hsc70, the form of Hsp70 expressed under normal growing conditions (25), is among the molecular chaperones whose levels are increased in thermotolerant cells (3, 24). Therefore, we determined the delay in 53BP1 complex formation in two other cell systems with elevated expression of Hsc70, heat radiosensitization-resistant (HR-1) cells and cells infected with Hsc70 adenovirus constructs. In HR-1 cells (16), there is increased expression of Hsc70 due to gene amplification (26); the decrease in TER (Supplementary Table S1) was also associated with attenuation of the delay in 53BP1 complex formation (Fig. 4B). Thus, we have shown by two different approaches (thermotolerant cells and HR-1 cells), that there was a correlation between the magnitude of the delay in 53BP1 complex formation and TER (in control HA-1 cells, thermotolerant cells and HR-1 cells, with a linear correlation with $R = 0.9986$ and $P < 0.001$; Supplementary Fig. S6). These results indicated that heat-induced delay of the formation of 53BP1 complexes either plays a direct role in heat radiosensitization, and/or is associated with a process involved in heat radiosensitization.

In order to further test the hypothesis that modulation of the levels of functional Hsc70 affects the heat-induced delay of 53BP1 complex formation, we took advantage of an adenosivus-based expression system of wild-type Hsc70 and a dominant negative mutant of Hsc70, D199S, which has an ATPase activity that is reduced by two orders of magnitude (23, 27). The ATPase activity of Hsc70 is crucial for its function (23, 27). Infection of HeLa cells with wild-type Hsc70 or the negative dominant mutant, did not alter the induction or resolution of 53BP1 complexes in cells treated with radiation alone (Fig. 4C). On the other hand, the expression of the wild-type Hsc70 construct significantly decreased the heat-induced delay of 53BP1 complex formation in heated-irradiated cells, whereas expression of the dominant negative Hsc70-D199S mutant considerably extended this delay (Fig. 4D). Thus, we have shown by three different approaches that Hsc70, an important molecular chaperone (23, 28), can modulate the heat-induced delay of 53BP1 complex formation in heated-irradiated cells.

53BP1 plays a role in heat radiosensitization. In order to further determine if 53BP1 plays a role in heat radiosensitization, we turned to 53BP1 knockout MEFs. There was no significant difference in the heat sensitivity of 53BP1$^{+/+}$ and 53BP1$^{-/-}$ MEFs (Fig. 5A), although there was a significant difference in their radiation sensitivity (Fig. 5D), as described previously (15, 20). Heat radiosensitization was observed in both 53BP1$^{+/+}$ and 53BP1$^{-/-}$ MEFs; however, the TER was attenuated in the 53BP1$^{-/-}$ MEFs (Supplementary Table S2). The attenuation of TER in 53BP1$^{-/-}$ MEFs suggested, by using an epistatic argument, that 53BP1 either plays a direct role in heat radiosensitization or is a surrogate marker for heat-induced alterations in DNA DSB signaling pathways involved in heat radiosensitization. Because the epistatic interaction was not complete (i.e., heat radiosensitization was still observed in the 53BP1$^{-/-}$ MEFs), heat effects on other components of the cellular response to IR-induced DNA DSB may also play a role in heat radiosensitization. The 53BP1$^{-/-}$ MEFs are the first radiosensitive cell line examined to date that displays reduced heat radiosensitization, except for ataxia-telangiectasia (AT) cells grown under specific conditions (29).

Effects of heat on IR-induced events downstream from 53BP1 complex formation. Knocking down or knocking out 53BP1 has indicated that several processes involved in the cellular response to IR-induced DNA damage, including the phosphorylation of SMC1 and Chk2 and the formation of foci containing them, are events downstream from 53BP1 (14, 15, 30). For further validation of the conclusion that 53BP1 plays a role in heat radiosensitization, we ascertained the effect of heat on events downstream from 53BP1. Heat alone did not induce foci containing Chk2 p53T (Supplementary Fig. S7A and B). The formation of such foci was delayed in heated-irradiated cells (Fig. 6A and B). Similar results were obtained with the formation of foci containing Chk2 p68T (Supplementary Fig. S7A and B). The heat-induced delay in these two processes downstream from 53BP1 in heated-irradiated cells paralleled the heat-induced delay in 53BP1 complex formation (Fig. 1C).

Discussion

In this study, we found that heat-induced alterations in the early phase of cellular response to IR-induced DNA damage are
associated with heat radiosensitization. Specifically, heat induces a delay in 53BP1 complex formation in heated-irradiated cells. We have generated several lines of evidence that 53BP1 plays a role in heat radiosensitization. The formation of the 53BP1 complex is delayed in heated-irradiated cells. The magnitude of the delay in 53BP1 complex formation is attenuated in thermotolerant and HR-1 cells. There is a direct correlation between TER and the length of the heat-induced delay in 53BP1 complex formation; DNA damage response signaling events downstream from 53BP1 are delayed in parallel with the heat-induced delay in 53BP1 complex formation. Finally, heat radiosensitization is attenuated in 53BP1-null cells, in contrast with other radiosensitive cell lines that have been tested (5). Taken altogether, these results strongly support a role for 53BP1 in heat radiosensitization. However, our data do not exclude the possibility that 53BP1 is a surrogate reporter for heat-induced perturbations of other facets of the cellular response to DNA damage set in motion by IR. Nevertheless, our results do support the conclusion that the sensing and transducing mechanisms involved in the cellular response to IR-induced DNA damage are affected by the heat-induced alterations in protein-protein interactions, in addition to blocking access of the repair machinery to its template (1, 4).

How might 53BP1 play a role in heat radiosensitization? We propose that during the heat-induced delay in 53BP1 complex formation in heated-irradiated cells, the cells display a transient "phenocopy" of 53BP1−/− cells or 53BP1 knockdown cells. This notion is supported by the observations that the perturbations of steps in the response to IR-induced DNA damage downstream from 53BP1 found in such cells, such as the phosphorylation of T68 of Chk2 and S957 of SMC1 (14, 15, 30), are observed during the delay of the formation of the 53BP1 complex in heated-irradiated cells (Fig. 6; Supplementary Fig. S7). It is of interest to note that both Chk2 and SMC1 are substrates of ATM (31). Indeed, 53BP1 has been postulated to be involved in regulating ATM signaling (14, 32). Thus, the heat-induced perturbations of radiation-induced ATM signaling that we have reported earlier (13) may be mediated, at least in part, through interference with the formation of the 53BP1 complex.

**Figure 4.** Delay of 53BP1 complex formation in cells exposed to various experimental conditions. A, control (C), and transiently thermotolerant (TT; cells heated at 43°C for 30 min and allowed to recover at 37°C for 24 h) HA-1 cells were irradiated with 2 Gy of X-rays alone (■, ●) or irradiated with 2 Gy of X-rays and then heated at 43°C for 60 min (▲, ●), then stained for 53BP1 foci at various times posttreatment and the fraction of cells with more than four 53BP1 foci was determined. B, wild-type HA-1 and heat radiosensitization–resistant HR-1 cells were irradiated with 2 Gy of X-rays alone (■, ●) or irradiated with 2 Gy of X-rays followed by treatment at 43°C for 60 min (▲, ●), then stained for 53BP1 foci at various times posttreatment and the fraction of cells with more than four 53BP1 foci was determined. C, control HeLa cells (○), HeLa cells infected with a wild-type Hsc70 adenovirus construct (◆), and HeLa cells infected with the mutant D199S Hsc70 adenovirus construct (▲) were all irradiated with 2 Gy of X-rays, then stained for 53BP1 foci at various times postirradiation and the fraction of cells with more than six 53BP1 foci was determined. D, control HeLa cells (○), wild-type Hsc70 adenovirus infected HeLa cells (◆), and D199S mutant Hsc70 adenovirus construct infected HeLa cells (▲) were irradiated with 2 Gy of X-rays followed by a 60-min treatment at 43°C, and were stained for 53BP1 foci at various times posttreatment and the fraction of cells with more than six 53BP1 foci was determined.
53BP1-null mice are radiosensitive and have a compromised immune system (15). The mechanism of radiosensitivity in 53BP1−/− MEFs has not been elucidated; however, long-term DNA damage, as assayed by the persistence of γ-H2AX foci, is involved (21). The immune deficiency in 53BP1-null mice results from impairment of the B cell–specific process of IgH class switch recombination (CSR; refs. 14, 33, 34). CSR is an inducible event that occurs at a defined chromosomal location, the S locus on mouse chromosome 12 (14, 35). The DNA DSB repair defect in CSR occurs at the level of synopsis or DNA end rejoining (34, 35). γ-H2AX has been implicated in tethering together broken chromosome ends through long-range synopsis at the switch regions (14, 35), leading to the proposal that γ-H2AX participates in an end-rejoining process that is distinct from classic NHEJ, called “anchoring” (36). 53BP1 has been proposed to have a similar anchoring function (34, 37). In this capacity, both γ-H2AX and 53BP1 facilitate the repair of DNA DSB at broken chromosomes by anchoring DNA ends in preparation for end processing and ligation by the NHEJ machinery (14, 34–37).

Although the anchoring function has been proposed for 53BP1 in CSR, it is possible to envision a scenario in which 53BP1 performs a similar function in other cell types in response to DNA damage. Indeed, 53BP1 has a very specific anchor in chromatin, H4-K20 Me (2, 38), and the putative XRCC4-dependent function of 53BP1 in chromosomal NHEJ (39) is strictly dependent on the interaction of 53BP1 with its chromatin anchor. Thus, in heated-irradiated cells, the transient lack of the anchoring function of 53BP1 could explain the increase in the fraction of slowly repaired DNA DSB associated with heat radiosensitization (1, 5). The period of increase of this fraction of radiation-induced DNA DSB (40, 41) corresponds with the period of delay in 53BP1 complex formation (Fig. 1B). The increase in the relative fraction of slowly rejoined IR-induced DNA DSB leads to an increased probability for the persistence of unrejoined DNA DSB and/or the generation of misrejoined DNA DSB, both leading to lethal chromosomal aberrations (1, 5, 42). In H2AX−/− MEFs, it has been shown that the initial recruitment of 53BP1 to sites of DNA damage is independent of H2AX, whereas the persistence of 53BP1 IRIF is dependent on H2AX. In the H2AX−/− MEFs, 53BP1 IRIF are present for 30 to 60 minutes after irradiation, after which they are dispersed (10). It is well accepted that >90% of IR-induced DNA DSB are repaired within 60 minutes postirradiation (1, 40, 41). The majority of 53BP1 IRIF are resolved within 6 to 8 hours of irradiation in cells expressing H2AX, such as the cells used in this study (Figs. 1C and 4C). Thus, the presence of 53BP1 IRIF for 6 to 8 hours may be required for the optimal efficiency of DNA DSB rejoining events that occur with slow kinetics, perhaps the type of DNA DSB that require more end processing. Therefore, the overall results presented above support the notion that the heat-induced delay in 53BP1 complex formation leads to perturbations in the repair of DNA DSB that are rejoined slowly (40, 41).

The ability of the molecular chaperone Hsc70 to modulate the heat-induced delay in 53BP1 complex formation is intriguing. The delay is modulated in both heat radiosensitization–resistant cells (16) that overexpress Hsc70 due to gene amplification (ref. 26; Fig. 4B) and in cells in which Hsc70 is overexpressed using an adenovirus construct (ref. 23; Fig. 4C). In both cases, the delay is attenuated, whereas in cells that express a negative dominant mutant form of Hsc70, the delay is extended (Fig. 4D). The Hsc70 protein is known to protect native protein structures by inhibiting protein aggregation and by refolding unfolded proteins (28, 43). The site of action for the Hsc70-dependent modulation of heat-induced delay in the formation of the 53BP1 complex is not clear at this time because the mechanism(s) involved in this delay are yet to be elucidated. However, it is of interest to note that we have shown that heat does not lead to a global insolubilization of 53BP1 (Fig. 3), indicating that the protective action of Hsc70 may occur at a heat effect on 53BP1 complex formation distinct from the aggregation of or the refolding of 53BP1. Because the assembly of the 53BP1 complex requires the close interaction of several newly identified proteins in addition to γ-H2AX and MDC1 (44–46), the chaperoning function of Hsc70 could modulate heat effects on these novel interactions.

Conceptually, there are two possibilities for the site of action of heat in the delay of 53BP1 complex formation: the γ-H2AX/MDC1 complex or 53BP1 itself. On one hand, the heat-induced γ-H2AX/MDC1 complexes may interfere with the formation of the IR induced γ-H2AX/MDC1 complexes and/or the heat-induced γ-H2AX/MDC1 complexes may be different than their radiation-induced counterparts.1 Recently, it has been shown that the phosphorylation of MDC1 at T699, T719, T752, and T769 by ATM and ubiquitinylation of H2AX through the action of RNF8 and

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1. A. Laszlo and I. Fleischer, unpublished observations.
Ubc13 are required for the formation 53BP1 IRIF (44–46). The effect of heat on these upstream steps in the formation of 53BP1 IRIF remains to be determined. On the other hand, heat could have a direct effect on 53BP1. As mentioned previously, heat did not induce a global insolubilization of 53BP1 (Fig. 3). Nevertheless, heat could affect the correct formation of 53BP1 multimers (47), which has been shown to be required for complementation of the radiosensitivity of 53BP1−/− MEFs, because their transfection with a 53BP1 construct defective in multimer formation failed to restore radioresistance (21). At the present time, the possibility that heat affects both the γ-H2AX/MDC1 complex and 53BP1 itself cannot be eliminated.

In a recent report, we have shown that a novel heat enhancer reduces the threshold for both heat radiosensitization and the heat-induced delay in 53BP1 complex formation (48). Thus, rapid assays using the heat-induced delay of 53BP1 complex formation as a readout could be used to screen for heat enhancers and heat mimics.

In conclusion, we have shown that in addition to interfering with the availability of chromatin to the repair machinery of DNA DSB (1, 4, 5), heat also disturbs a specific step in the one of the earliest steps of the cellular response to DNA DSB, i.e., the formation of the 53BP1 complex. We have shown that such perturbations are good candidates for playing a pivotal role in heat radiosensitization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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