Uncoupling of M-Phase Kinase Activation from the Completion of S-Phase by Heat Shock


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ABSTRACT

Chronic exposure of asynchronous HeLa cell cultures to 41.5°C leads to an accumulation of cells in the S-phase, spontaneous premature chromosome condensation, and loss of clonogenicity (M. A. Mackey, S. L. Anolik, and J. L. Roti Roti. Cancer Res., 52: 1101–1106, 1992). In this report, we show that increases in histone H1 kinase activity during 41.5°C exposure occur coincidentally with the appearance of premature chromosome condensation. Furthermore, this kinase activity is shown to be associated with M-phase kinase complexes containing cyclin B1. These increases in the activity of M-phase kinase were found to occur concomitantly with an elevation in cyclin B1 mRNA and an accumulation of cyclin B1 protein. Because cyclin B1 transcription begins in the S-phase, it is probable that the heat-induced delay in the S-phase allows the accumulation of abnormally high cyclin B1 levels. Elevated cyclin B1 levels could then account for the observed abrogation of the cell cycle checkpoint, which usually assures that mitosis does not proceed until DNA replication is complete. This involvement of M-phase kinase in heat-induced cytotoxicity demonstrates the importance of the coordinate regulation of the processes of DNA replication and entry into mitosis.

INTRODUCTION

Specific molecular pathways have evolved to prevent cell division prior to the completion of DNA replication (1). When G2 cells are fused with S-phase cells, mitosis is delayed in the heterokaryon until replication is complete in the S-phase nuclei (2). In Xenopus cell-free systems, events associated with mitosis, such as M-phase kinase activation, exhibit oscillatory behavior, even in the absence of DNA. The addition of unreplicated DNA above a certain threshold moment delays these oscillations in a dose-dependent manner, suggesting a role for unreplicated DNA in the negative feedback regulation of M-phase kinase activation (3). The cdc25 gene product has been identified as the enzyme responsible for the dephosphorylation-mediated activation of the p34cdc2 moiety in M-phase kinase complexes, and the wee1 and mkl1 gene products phosphorylate this same residue, thus acting as negative regulators of M-phase kinase activation (1). Uncoupling of the onset of mitosis from the completion of DNA replication has been observed in vivo in organisms in two different circumstances: (a) fission yeast cdc2-F159 mutants, in which tyrosine 15 has been replaced with a phenylalanine residue, which cannot be phosphorylated, thus abrogating the cdc25-mediated control of M-phase kinase activation (4); and (b) baby hamster kidney cells arrested in the S-phase using DNA synthesis inhibitors in the presence of caffeine (5). A study conducted using Xenopus cell-free systems containing unreplicated DNA established that caffeine-induced, premature mitotic events were associated with activation of the cyclin B1-p34cdc2 complex, not with aberrant cyclin or p34 synthesis or accumulation (3). This finding led the authors to suggest that caffeine acts by interfering either specifically or nonspecifically with phosphatases or kinases, which, among other functions, act to regulate M-phase kinase activation.

This report describes the untimely activation of M-phase kinase while HeLa cells are delayed in the S-phase during a long-duration treatment at 41.5°C. Data are presented that implicate an abnormal accumulation of cyclin B1 in kinase activation under these conditions. Because we have previously shown a correlation between the incidence of spontaneous premature chromosome condensation and cell killing in this experimental system (6), it is possible that excess cyclin B1-driven, premature mitotic events may underlie this cell lethality. Presumably, cell division prior to the completion of DNA replication would represent a lethal event, thus requiring strict regulation in cellular organisms (1). The clinical application of hyperthermia may benefit from such knowledge of molecular mechanisms of heat-induced lethality.

MATERIALS AND METHODS

Cell Culture. HeLa S3 cells were grown at 37°C in a suspension culture in Joklik-modified MEM supplemented with 7% iron-supplemented calf serum containing antibiotics (500 units/ml sodium penicillin G and 500 μg/ml streptomycin sulfate). Cells were maintained in the suspension culture at 1–3 × 10^5 cells/ml. All experiments were performed with a starting cell density of 10^5 cells/ml.

Heat Treatment. Cells were heated in the suspension in a Thelco incubator (Precision Scientific Co.), which was modified in our laboratory for these experiments, as described previously (6).

Determination of Mitotic-like Cell Fraction and Cell Cycle Phase Distribution. For determination of the fraction of cells exhibiting SPCC, the acid-AO flow cytometric assay was used, as modified (7) at our institution from the method of Darzyynkiewicz. At the indicated times, 10–100 cells/sample) samples were centrifuged (400 × g for 5 min at 4°C) and fixed in 70% ethanol overnight at 4°C. Prior to analysis, samples were washed twice in PBS, and the resuspended pellet was incubated with 50 μg/ml recently boiled RNase A (Sigma Chemical Co.) for 30 min at 37°C. To each 0.2-mll sample containing 2 × 10^5 cells, 0.5 ml acidic solution (0.1 M KC1 and 0.1 M HCl (pH 1.4)) was added. After 45 s of denaturation at room temperature, 1 ml AO solution (10 μg/ml AO in 0.1 M citric acid and 0.1 M NaPO₄ (pH 2.6) was added, and the sample was ready for flow cytometric analysis.

For anti-BrdUrd-propidium iodide cell cycle phase percentage determinations, 10 ml cell suspension (~10^6 cells/sample) were incubated in medium containing 10 μM BrdUrd (Sigma) for 30 min at 41.5°C, followed by centrifugation, fixation, and storage as above for the acid-AO assay. After two PBS washes of the fixed cell samples as above, the samples were treated with 5 ml 0.4 mg/ml pepsin (Sigma) in 0.1 M HCl for 10 min, followed by a PBS wash. The pellet was then resuspended in 5 ml 2 N HCl and incubated for 30 min at room temperature, followed by centrifugation as before and resuspension of the pellet in 5 ml 0.1 M NaOPO₄ (pH 8.5) to neutralize the acid. After centrifugation as before, the pellet was resuspended in 0.05 ml PBT [0.5% Tween 20 and 1 mg/ml BSA (Sigma) in PBS (pH 7.2)]. Anti-BrdUrd antibody (0.005 ml; Becton Dickinson) was then added, and room temperature staining was allowed for 30 min in the dark, followed by a 1-ml wash with PBT and centrifugation as before. After resuspension of the pellet in 0.05 ml PBT, 0.005 ml fluorescein-conjugated second antibody (affinity-purified rabbit antirabbit

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3 The abbreviations used are: SPCC, spontaneous premature chromosome condensation; AO, acridine orange; BrdUrd, 5-bromodeoxyuridine; PBT, phosphate-buffered BSA-Tween 20; CHO, Chinese hamster ovary.
IgG; Sigma) was added, followed by 30 min of staining at room temperature in the dark. Following a final 1-m1 PBT wash and centrifugation, 0.1 ml recently boiled 1 mg/ml RNase A in PBS was added. After RNase digestion for 30 min at 37°C, 1 ml 20 μg/ml propidium iodide (Sigma) PBS was added, and the samples were allowed to stain for 30 min at room temperature, followed by flow cytometric analysis.

Flow Cytometry. Flow cytometric analysis was accomplished using a FACS IV cytometer (Becton Dickinson). Excitation was accomplished by the use of an argon laser emitting at 488 nm with 300-mW power. Red fluorescence was detected using a 640-nm low-pass filter, and green fluorescence was detected using a 525-nm band-pass filter. Data acquisition was triggered on the data analysis system (Cytomation, Inc.), interfaced to an IBM-compatible personal computer.

SDS-PAGE and Immunoblotting. One-dimensional SDS-PAGE was performed using standard techniques and 12.5% acrylamide gels. For experimental determination of kinase activity, following electrophoresis using 11-cm gels, samples were stained using Coomassie blue G-250, destained overnight, dried, and processed for autoradiography. Immunoblots were prepared using a minigel apparatus, according to the procedure of the manufacturer (Bio-Rad). Antibodies to cyclin B1 and cdc2 were obtained from Oncogene Science and used at the concentrations recommended by the manufacturer. Immunostaining was visualized using alkaline phosphatase-conjugated secondary goat antimuscle Fc antibodies (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies, Inc.). For quantification, laser densitometry (Molecular Dynamics Corp.) was used, scanning either the autoradiogram or the stained immunoblot.

Native Immunoprecipitation. To recover M-phase kinase activity, immunoprecipitation was performed under nonnading conditions, as modified from Druetta et al. (8). For each sample, 5 × 10⁶ cells were washed once with PBS, and the pellet was resuspended in 0.100 ml buffer A [80 mM glycerophosphate, 15 mM MgCl₂, 15 mM EGTA, 10% glycerol, 1% NP-40, and 0.1% 2-mercaptoethanol (pH 7.3); Ref. 9], followed by incubation on ice for 30 min and storage at −80°C until immunoprecipitation. Immunoprecipitation was performed using 100 μg protein (about 0.05 ml cell lysate, diluted 1:1 with buffer A after centrifugation of cell lysates in a microcentrifuge for 20 min at 4°C). Three μl of the appropriate antibody (either anti-cdc2 or anti-cyclin B1, as indicated in the figure legends) were then added, and the mixture was incubated for 1 h at 4°C. Next, 0.04 ml packed protein A-Sepharose in 0.5% BSA in PBS (preswollen overnight) was added, and samples were incubated for 1 h at 4°C. Immunoprecipitates were then washed twice using buffer A and twice using buffer B [50 mM Tris, 10 mM MgCl₂, 1 mM DTT, 20 μg/ml each leupeptin, pepstatin, chymostatin, aprotinin, and antipain, and 2 mM phenylmethylsulfonyl fluoride (pH 8.0)] and were then used in the kinase assay (see below).

M-Phase Kinase Assay. After immunoprecipitation, samples were incubated in buffer A containing 50 μg/ml purified histone H1 (Boehringer Mannheim) and 500 nm CAMP-dependent kinase inhibitor for 30 min on ice. Kinase activity of samples was then determined by the addition of [γ-³²P]ATP (185 kBq) made up to 25 μM in cold ATP after a 5-min preincubation at 30°C. Phosphorylation was allowed to proceed at 30°C for 10 min, after which the reaction was terminated using hot SDS-PAGE sample buffer, and 12.5% SDS-PAGE was used to resolve the phosphorylated Histone H1.

Northern Blots. Samples for Northern blot analysis were prepared by centrifugation (400 × g at 4°C) of 50 ml cell suspension (1–2 × 10⁶ cells), followed by resuspension of cell pellet in 0.5 ml TRI reagent (Molecular Research Center, Inc.) for lysis. RNA purification was performed according to the procedure of the manufacturer; quantification of total RNA was performed spectrophotometrically. For each sample, 10 μg total RNA were run on a 1% agarose-formaldehyde gel. Northern transfer onto a Duralon UV membrane was as described by Maniatis et al. (10). The transferred RNA was cross-linked to the membrane using a Stratalinker-1800 (Stratagene). The cyclin B1 cDNA clone (11) was obtained from Dr. Gilles McKenna (Department of Radiation Oncology, University of Pennsylvania), with the permission of Dr. Tony Hunter. The 18S rRNA cDNA clone was obtained from the American Type Culture Collection. A 1.5-kb BamHI fragment of the cyclin B1 cDNA and a 1.5-kb EcoRI fragment of the 18S rRNA cDNA were used as probes for the Northern blot analysis. Probes were radiolabeled using the Multiprime DNA labeling system (Amersham) and purified using Sephadex G50 columns.

hybridization condition was 10% dextran sulfate/1 mM NaCl/1% SDS at 65°C overnight. Following hybridization, membranes were washed twice in 2× SSC [1× SSC = 0.15 M NaCl and 0.015 M Na citrate (pH 7.0)]/1% SDS at 65°C then autoradiographed at −80°C with an intensifying screen. Quantification was obtained using a laser densitometer (Molecular Dynamics).

RESULTS

When HeLa S3 cells were incubated at 41.5°C, cells were arrested in the S-phase (Fig. 1), with significant accumulation of cells in the S-phase occurring after 12-h treatment. In these experiments, the cell number did not change throughout the course of the hyperthermia treatment (data not shown). Note that the technique used for cell cycle analysis detected S-phase cells through BrdUrd labeling, thus demonstrating that significant DNA synthesis was occurring in these arrested cells, although probably at a reduced rate compared with unheated cells. As reported previously (6), heated cells seemed to arrest in late S-phase (data not shown), as evidenced by an accumulation of labeled cells with a near G₂ DNA content. BrdUrd-propidium iodide bivariate flow cytometric analysis was used to distinguish between cells in late S-phase and G₂, because univariate analyses (such as those obtained using only propidium iodide) do not. Following redistribution of cells into the S-phase, SPCC occurred, which has been previously shown to correlate well with a subsequent loss of cloning efficacy (6). The technique used to quantify SPCC, the acid-AO flow cytometric assay, takes advantage of the known resistance of chromatin in mitotic cells to acid hydrolysis to identify cells that contain mitotic-like chromatin condensation (7).

Concomitant with the appearance of SPCC, increases in histone H1 kinase activity were observed (Figs. 2 and 3). This increase in kinase activity closely paralleled the appearance of SPCC and was correlated temporally with the accumulation of cells in the S-phase. It is possible, based on these data alone, that cells could be in G₂ at the time of SPCC, although the persistence of the elevated S-phase fraction, along with the lack of a significant increase in the G₂ fraction, argues against the progression of cells out of the S-phase during a treatment, which results in a complete cessation of increases in cell number. Nevertheless, further study is needed to determine the exact cell cycle phase location at the time of SPCC under these conditions.

Cyclin B1-associated histone H1 kinase activity is required for entry of cells into mitosis. In the absence of perturbation, activation of this kinase activity is specifically inhibited by biochemical mechanisms dependent on the cdc25 and wee1 gene products (1). Histone
H1 kinase activity is frequently assayed by immunoprecipitation using an antibody to the cdc2 subunit of kinase complexes, as we did for the data shown in Fig. 2. Because the cdc2 kinase subunit can form complexes with other cyclins, notably cyclin A, interpretation of increases in anti-cdc2-precipitable histone H1 kinase activity as being reflective of increases in M-phase kinase activity must be confirmed by techniques specific to M-phase kinase activity. Accordingly, we examined kinase activity in cellular extracts that were immunoprecipitated using an antibody specific to cyclin B1, an accessory protein that is a required component of the M-phase kinase complex and has not been found to be associated with other cell cycle checkpoint mechanisms. Increases in kinase activity attributable to cyclin B1-containing complexes were evident in complexes precipitated from extracts prepared from cells heated at 41.5°C (see Fig. 4). Because M-phase kinase activity is required only for entry into mitosis, the persistent elevation in the SPCC fraction seen in Fig. 3, in light of the observed decrease in kinase activity shown in the same figure, probably reflects cells that have entered but are delayed in the exit of such aberrant mitoses. Under these conditions, mitotic cells present marked abnormalities, including a loss in the organization of cellular organelles and a disruption of nuclear structure, as determined in electron microscopic studies (12). It is probable that these alterations prolong the duration of these aberrant mitoses, thus leading to persistently elevated levels of SPCC.

For the experiments shown in Fig. 4, a slightly longer time at 41.5°C was required before M-phase kinase activity was observed to increase, and the increased kinase activity persisted throughout the 32-h experiment. In these experiments, this phenomenon was observed to be accompanied by a similar delay in the onset of SPCC induction (data not shown). It is not clear why the H1 kinase activity stayed elevated in these experiments (e.g., Fig. 4), whereas a decrease was usually observed at 24 and 28 h (as in Fig. 2); it is possible that these differences are attributable to slight differences in the cell cycle responses of these cell populations to the temperature perturbation, duly magnified by the long duration of the heating interval. In support of this interpretation is the fact that synchronized S-phase CHO cells exhibited alterations in cell cycle progression at temperatures in this range and were quite sensitive to small changes in temperature (13). Nevertheless, in the present studies, we found that the kinetics shown in Figs. 1–3 was most often observed in HeLa cells heated at 41.5°C. To avoid problems in data interpretation, we made simultaneous measurements of kinase activity, cell cycle progression, SPCC fraction, mRNA, and protein levels within each repeated experiment to arrive at the conclusions presented herein. For ease in data interpretation, the data shown in Figs. 1–3, 5, and 6 are from a single representative experiment.

It has been observed that cyclin B1 can drive the activation of the M-phase kinase stimulatory cdc25 phosphatase activity (14). Therefore, it is of interest to determine whether cyclin B1 levels are elevated during a 41.5°C exposure, which leads to kinase activation. Concomitantly with the increase in H1 kinase activity, a 5-fold increase in steady state cyclin B1 mRNA was found (Fig. 5), along with a corresponding increase in cyclin B1 protein, without any significant alteration in cdc2 levels (Fig. 6). These data thus implicate increased synthesis and accumulation of cyclin B1, occurring during the hyperthermia-induced delay in cell cycle transit, in the induction of SPCC. The decreases in cyclin B1 message and protein levels, which occur toward the end of these experiments, may reflect the entry into, and progression at least partly through, mitosis of the heated cells, because cyclin B1 is rapidly degraded under normal conditions toward the end of mitosis (1). Furthermore, mRNA synthesis is generally lower in mitotic cells, consistent with the increased condensation state of mitotic chromatin. However, transcriptional rate studies, using nuclear run-on assays, would be needed to verify this contention.

**DISCUSSION**

Clinical experience with the use of hyperthermia as a cancer treatment modality has shown that intratumoral temperatures that can be achieved are in the range of 41.5–42°C (15). Although rodent cell lines are refractory to cell killing in this temperature range, our work (6) and that of others (16, 17) has demonstrated that many human cancer cell lines exhibit lethality during prolonged exposure to this range of treatment temperatures. This report presents evidence that lethality under these conditions of long-duration heat treatment may be associated with observed cell cycle alterations, in that abrogation of the G2-M cell cycle checkpoint allows an untimely mitosis and, subsequently, cell death. Cells that are delayed in passage through the
S-phase, presumably due to the heat-induced inhibition of DNA synthesis, undergo M-phase kinase activation, SPCC, and extensive nuclear fragmentation. These events are not associated with apoptosis in the cell line studied and may represent a novel mode of cell death (12). Knowledge of the molecular mechanisms underlying such premature entry into mitosis before the completion of DNA replication may further provide insights into mechanisms of cell cycle regulation.

cdc25 activity is thought to be a regulator of M-phase kinase activity, ensuring that mitosis does not proceed before the completion of DNA replication (1). It has been shown that elevated levels of cyclin B1 can lead to the activation of the cdc25 gene product in vitro (14). It is possible that, in a cell, when the pool of inactive M-phase kinase complexes accumulates above some threshold level, regulation of M-phase kinase activation by both cdc25 and wee1 (and mik1, presumably) may be impeded. Thus, the heat-induced, abnormal accumulation of cyclin B1 in cells delayed in the S-phase may lead to M-phase kinase activation prior to the completion of DNA replication. Because cyclin B1 is preferentially synthesized in late S-phase and G2 in HeLa cells (11), it is likely that this accumulation of cyclin B1 mRNA and protein is a direct consequence of the heat-induced delays in S-phase transit. To test this hypothesis, it might be useful to determine whether blockage of cells in late S-phase using DNA synthesis inhibitors would lead to overaccumulation of cyclin B1 and subsequent SPCC.

In CHO cells, arrest of cells in the S-phase after an acute heat exposure has been associated with DNA synthesis inhibition (18), and such inhibition has been implicated in the observed induction of chromosome aberrations in heated S-phase cells at this temperature (19). It is possible that lethal processes similar to those described in this report are mechanisms whereby cells containing incompletely replicated or damaged DNA are removed from the population, thus helping ensure the fidelity of genetic inheritance in the face of environmental stress.

Another possible explanation for the observed M-phase kinase activation relies on evidence from genetic studies in yeast (20). When deletion mutants were constructed with defective wee1 and cdc25 genes, SPCC was observed. Thus, it is possible that the overabundance of cyclin B1 protein in cells heated under these conditions in effect may dilute the available cdc25 and wee1 gene products and thus may lead to kinase activation. Alternatively, such activation may be attributable to nonspecific induction of phosphatase activity during the long-duration heat treatment. This explanation, however, is less favorable, due to the complex regulation of cdc2, which is known to be mediated by its phosphatolation state. Thus, dephosphorylation would, in addition to activation of M-phase kinase activity when occurring at tyrosine 15 residues of the cdc2 moiety, lead to inhibition of the kinase complex if dephosphorylation were to occur at threonine 161 of cdc2.

An earlier comparison of the effects of long-duration 41.5°C exposure on CHO cells with those observed using HeLa cells demonstrated that CHO cells were blocked in G1 during the heat treatment, whereas the human cancer cell line redistributed into the S-phase, underwent SPCC followed by nuclear fragmentation, and subsequently died (6). Associated with the G1 arrest of CHO cells was a reduction in both SPCC induction and cell killing, implicating progression into and through the S-phase during the heat treatment in the observed cytotoxicity of hyperthermia under these conditions. The present study suggests that the delay in cell cycle transit through the S-phase in HeLa cells occurring during a
long-duration 41.5°C exposure leads to an abnormal accumulation of cyclin B1, and that such accumulation leads to the untimely activation of M-phase kinase in S-phase-arrested cells and, ultimately, cell death.

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