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Histone H1 and Cdk1 Kinase Activities in Early Embryos of Four Mouse Strains after X-irradiation

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Abstract. *The cdk1/cyclin B1 complex is a universal regulator known to be responsible for driving the cell-cycle from the G2- to the M-phase. To investigate the effects of irradiation on the activity of this complex in preimplantation embryos, we irradiated one- and two-cell mouse embryos with X-rays, and measured the fluctuations of histone H1 and cdk1 kinase activity. Four mouse strains with different radiation sensitivities were chosen: the BALB/c and the Heiligenberger (radiation-sensitive) and the C57BL and the CF1 (radiation-resistant) strains. Embryos irradiated in the first cell-cycle arrested in the G2-phase. However, the dynamics of this radiation-induced G2-block were different between the mouse strains tested. Indeed, in the C57BL and the CF1 strains, X-irradiation with 2.5 Gy induced a very short G2 block before the one-cell embryos could then proceed to mitosis. On the contrary, X-irradiation in BALB/c induced a G2-arrest that lasted about 20 h, with the percentage of embryos blocked in G2 depending on the dose, whilst in the Heiligenberger strain, all irradiated embryos developed a G2-block, which was dependent in duration on the radiation dose. In all mouse strains, the histone H1 kinase activity remained low during the G2 arrest, while it showed values comparable to that of control embryos during mitosis. X-irradiation is known to induce a change in the phosphorylation state of the cdk1 protein kinase in adult somatic cells. In embryos from the BALB/c and C57BL strains, the histone H1 kinase activities were confirmed by the cdk1 phosphorylation pattern: the inactive and phosphorylated form of cdk1 was observed in G2 arrested 1-cell embryos, while the active and dephosphorylated form of cdk1 was present in dividing control and irradiated 1-cell embryos. X-irradiation at the 2-cell stage only induced a short G2-arrest in all tested mouse strains. In conclusion, cell-cycle effects in early embryos under normal conditions and after irradiation are strictly paralleled by changes in the activity of the central cell-cycle driving enzyme complex.*

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Irradiation of eukaryotic cells has been shown to induce slowing of cell progression. Indeed, reversible, dose-dependent delays at one or more points in the progression of irradiated cells through the cell-cycle have been found in a wide variety of mammalian cells (1). Knowledge of how the activity of genes involved in cell-cycle control is regulated by exposure to ionizing radiation is critical in order to understand the molecular mechanisms underlying radiation-induced cell-cycle delays. Previous investigations have described alterations in irradiated cells of the expression patterns and activity of several key cell-cycle regulators (2-4). Failure to repair such damage before replication of DNA and mitosis may result in cell death or segregation of DNA lesions that can initiate or contribute to the development of cancer or foetal malformations.

Progress through the cell-cycle is subject to finely tuned feedback control mechanisms in which protein kinase complexes, consisting of a cyclin-dependent protein kinase (cdk) as the catalytic subunit and a cyclin molecule as the regulatory subunit, play a central, decisive role (Figure 1). Activity of these protein kinase complexes can be regulated on three levels: initially by differential phosphorylation of the cdk, secondly by the turnover of the cyclin subunit, and lastly by the binding of inhibitor molecules to the complex. The cdk1 protein kinase is thought to be an essential cell-cycle regulator of all eukaryotic cells (5). In yeasts, cdk1, through its association with distinct cyclins, activates entry into both the S-phase and mitosis (6). In mammalian cells, cdk1 protein complexes with cyclin B and is essential for the

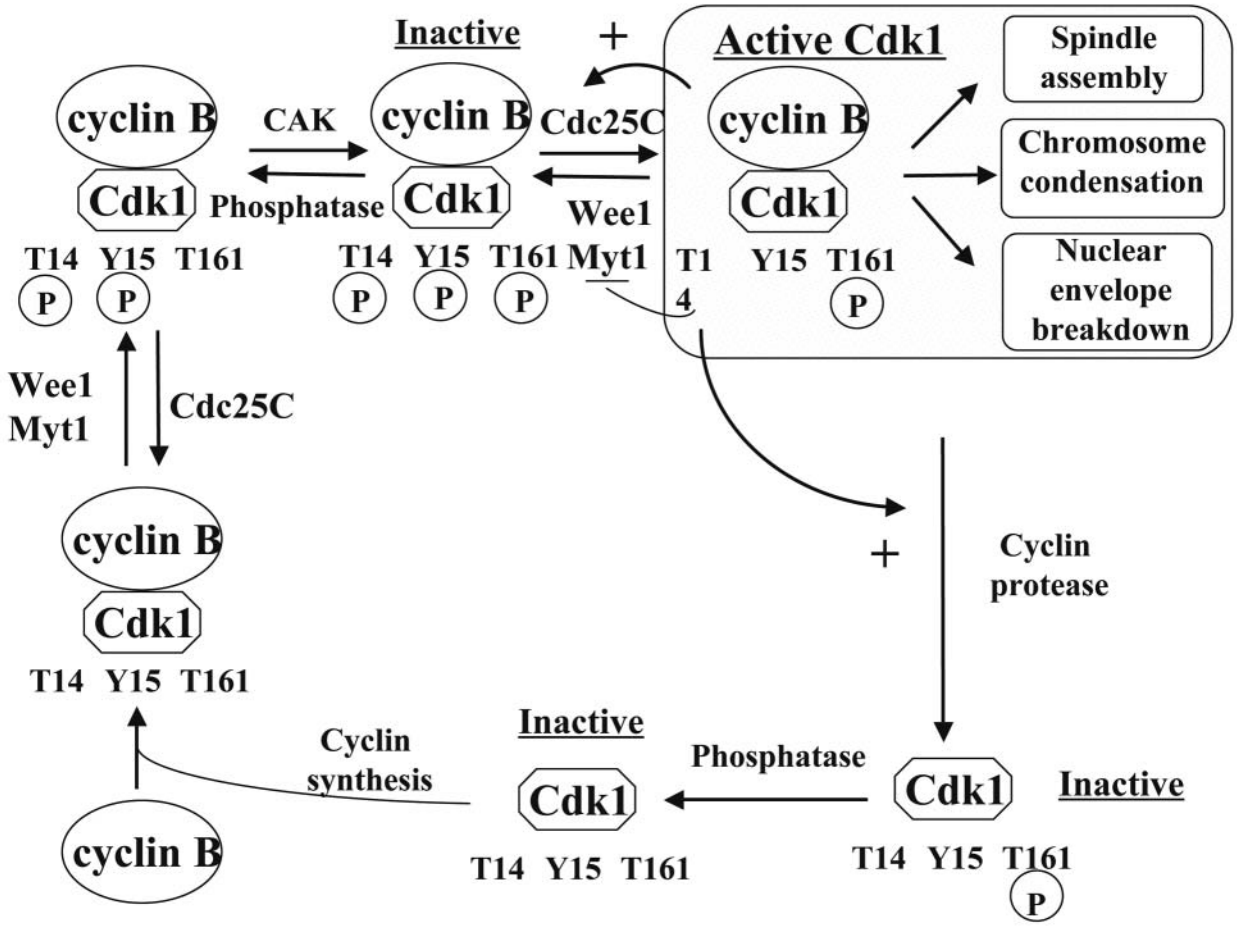

Figure 1. *Progression through G2- and M-phases is regulated by cdk1 kinase activity. This kinase activity is tightly regulated through two mechanisms. One is the binding of cdk1 to cyclins A and B, and the other is the phosphorylation of cdk1. Although cdk1 protein is present at essentially equal levels throughout the cell-cycle in growing cells, its kinase activity only appears in G2/M. Mitotic cyclin levels rise in S and G2. As cyclin levels rise and the G2/M transition nears, they bind with cdk1. While the binding of mitotic cyclins to cdk1 is necessary for its activation, it alone is not sufficient. The activity of cdk1 in mammalian cells is also regulated by phosphorylation of threonine 161, tyrosine 15 and threonine 14. The exact timing of the threonine 161 phoshorylation is undetermined, but evidence suggests that it may facilitate cyclin binding. Upon cyclin binding, cdk1 is also phosphorylated on threonine 14 and tyrosine 15 by regulatory kinase(s). The phosphorylation of tyrosine 15 is mediated by wee-1 kinase, but the kinase responsible for threonine 14 phosphorylation is currently unidentified. These phosphorylations inhibit cdk1 kinase activity. Final activation of the cyclin B-cdk1 complex is dependent upon dephosphorylation of both residues, which is mediated by the cdc25 phosphatase.*

G2/M-phase transition. Whereas cdk1 is present at a steady-state level during the cycle of continuously proliferating cells such as HeLa (7), it is induced with different kinetics during the stimulation of various quiescent cell types (8-9). After exposure of human tumor and diploid cells to ionizing radiation, changes in cdk1 activity have been observed and were found to be associated with altered patterns of phosphorylation of the cdk1 protein (10-11). Furthermore, studies with human pre-B leukaemia cells have shown that ionizing radiation promotes the physical interactions between cdk1 and the Src family protein-tyrosine kinase Lyn in the cytoplasm of the irradiated cells, leading to tyrosine phosphorylation of cdk1 (12). Moreover, radiation-induced binding of cdk1 to p56/p53 lyn kinase was associated with inhibition of cdk1 activity and has been reported in nuclear lysates of HL60 cells (10).

The mechanisms of cell-cycle regulation in early embryos are not fully understood. In mammalian germ cells, gene transcription ceases with oocyte maturation and is not switched on again until after fertilisation, in the second cell-cycle, or later, depending on the species. Therefore, cell-cycle analysis in early mouse embryos represents a unique opportunity to study cell-cycle regulation in a mammalian cell system with and without an actively transcribing genome. The aim of this study was to

extend the information on the effect of ionizing radiation on early embryos. For this purpose, four mouse strains were selected for their difference in radiation sensitivity and changes in histone H1 and cdk1 kinase activities were monitored after irradiation. Since the capacity of cdk1 to phosphorylate histone H1 is widely used to evaluate the protein kinase activity of the cyclin/cdk1 complex, the fluctuations of histone H1 kinase activity in control and irradiated embryos were evaluated using a method developed to measure histone H1 kinase activity in single oocytes, and was applied to one- and two-cell embryos. Ovulated oocytes that are in metaphase of second meiosis and therefore exhibit a high histone H1 kinase activity served as controls.

Materials and Methods

Animals, ovulation, embryo collection and irradiation. Female BALB/c, C57BL and CF1 mice aged 10-12 weeks were provided from B&K Universal (Hull, UK). Female Heiligenberger mice aged 10-12 weeks were kindly provided by Dr Wolfgang Müller, Essen, D. The mean weight of the females at the time of the experiment was around 30 g. Between 30 and 40 animals per X-irradiation condition and per mouse strain were used for these experiments. BALB/c, C57BL and CF1 females were induced to superovulate by intraperitoneal injection of 5 i.u. pregnant mare serum (PMS, Folligon®, Intervet, Beaucouse, France) followed 45-48 hours later by 5 i.u. human chorionic gonadotrophin (hCG, Pregnyl®, Organon, Brussels, Belgium). Superovulation occurs at 12±3 h after hCG injection (13). Since Heiligenberger females did not respond to superovulating hormones, oocytes and embryos from that strain originated from naturally ovulated animals. In order to obtain embryos, females were individually caged with males of the same strain from 15 to 17 h after hCG injection (BALB/c, C57BL and CF1 strains) or for two hours in the morning (Heiligenberger strain), and they were examined immediately thereafter for the presence of a vaginal plug. Fertilization of the positive females was considered to have occurred at the middle of this short mating period.

Pregnant females were whole-body irradiated with a Pantak HF420 RX machine (250 kV, 15 mA, 1 mm Cu filtration, dose-rate 0.375 Gy/min, Branford, CT, USA), 8 h after presumed fertilization. The dose applied (2.5 Gy) had been previously shown to induce a very strong G2-arrest in BALB/C zygotes (14). Doses of 0.5 Gy and 1 Gy X-ray were additionally used for the Heiligenberger strain.

Oocyte and embryo collection and culture. Superovulated oocytes in metaphase of second meiosis were collected by rupture of the ampulla tubae 15 h post hCG (BALB/c, C57BL and CF1 mice) in Yamada's medium containing 0.01 mM EDTA and 0.1% hyaluronidase (Sigma, Bornem, Belgium) to disperse follicular cells (15). Naturally ovulated oocytes from the Heiligenberger strain were collected in the same culture medium.

Embryos were flushed out from the ampulla tubae immediately after X-irradiation, in the same medium as that used for oocyte collection. Only those that had extruded the second polar body and formed two pronuclei were used for the experiments. Embryos were rinsed in hyaluronidase-free medium and cultivated at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Embryos were regularly examined under a phase contrast microscope for the rupture of the pronuclear envelopes (NEBD) and cleavage to the 2-cell stage, beginning at 22 h *p.c.* (post conception). In order to obtain embryos in the metaphase of either the first or second division, they were incubated in Yamada's medium supplemented with colchicine (250 ng/ml, Gibco BRL, Merelbeke, Belgium). Exposure to colchicine lasted from 8 h to 28 h *p.c.* for obtention of first mitosis (control and irradiated embryos from the C57BL, CF1 and Heiligenberger strains) and from 34 h to 48 h *p.c.* for obtention of second mitosis (control embryos) or delayed first mitosis (irradiated embryos from the BALB/c strain). Embryos were collected in mitosis or at different times during interphase and, as for oocytes in MII, they were assayed for histone H1 kinase activity and cdk1 kinase activity.

Histone H1 kinase activity assay. Oocytes and embryos at the 1- or 2-cell stage were pipetted and collected in 2 μL of an ice-cold collection buffer of phosphate-buffered saline (PBS) supplemented with 1% polyvinyl alcohol, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium orthovanadate ($NaVO_4$) and 10 mM sodium fluoride (16). The samples were frozen and thawed in liquid nitrogen in order to lyse cells and stored at –80˚C for a maximum of one week. Oocytes and embryos were analyzed for histone H1 kinase activity using histone H1 as the exogenous substrate, [γ-^{32}P]ATP to allow the phosphorylation of the substrate and protein kinase inhibitor peptide to block cyclic AMP-dependent protein kinase. The kinase reaction was performed as follows: 3 μL of HB buffer were added to each sample and the samples were incubated 15 minutes at 37˚C. The HB buffer contained 100 mM alpha-glycerophosphate, 25 mM p-nitrophenylphosphate, 25 mM $MgCl_2.6H_2O$, 1.3 mM dithiothreitol (DTT), 0.13 NaVO4, 1.33 mM phenylmethylsulfonyl fluoride (PMSF), 33 μg/mL leupeptin, 66 μg/mL aprotinin, 42 mM 3-[N-morpholino]propanesulfonic acid (MOPS-KOH) and 8.3 mM ethylene glucobis (alpha-aminoethyl ether) (EGTA). A volume of 4 μL of kinase buffer were added to each sample. The kinase buffer contained 75 mM alpha-glycerophosphate, 19 mM p-nitrophenylphosphate, 19 mM $MgCl_2.6H_2O$, 1.25 mM DTT, 0.125 mM $NaVO_4$, 1.25 mM PMSF, 25 μg/mL leupeptin, 50 μg/mL aprotinin, 31.25 mM MOPS-KOH pH 7.2, 6.25 mM EGTA, 2.5 mg/mL histone H1 type III from calf thymus, 1.25 μM protein kinase inhibitor peptide (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asu-Ala-Ile-His-Asp) and 1 μCi [γ-^{32}P]-ATP 167 TBq/mmol. The reaction was allowed to proceed for 30 min at 37˚C and then stopped by addition of 10 μL of SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.5% bromophenol blue) (17). All chemicals used in these experiments were purchased from Sigma and the [γ-^{32}P]-ATP compound from ICN Biomedicals (Brussels, Belgium).

After denaturing by boiling the samples for 3 min, the proteins were separated on 10% polyacrylamide gel (SDS-PAGE) in Mini Protean II cells (BioRad, Nazareth, Belgium) according to the method of Laemmli (17). The protein bands were visualized after Coomassie blue staining. Histone H1 kinase activity was revealed by autoradiography of the dried gels exposed for 4 h at –80˚C using Kodak X-OMAT AR films (Sigma, Bornem, Belgium). The bands corresponding to histone H1 were then excised from the gels and the incorporation of [γ-^{32}P]-ATP into histone H1 was determined by liquid scintillation counting in a Packard counter after adding 1 mL of water overnight, 1 ml of soluene-350 (Packard) at 50˚C for 3 h

and 9 mL of Instagel (Packard Instrument, Brussels, Belgium) to each sample. As a control for non-specific non-enzymatic labeling of histone H1, the complete kinase reaction was performed without embryos or oocytes and the background values obtained were subtracted from each sample. The incorporation of [γ-^{32}P]-ATP into histone H1 was expressed as counts per minute (cpm) per embryo or per oocyte. Further details of the histone H1 kinase assay can be found in Baatout *et al.* (18, 19).

Determinations of the state of phosphorylation of cdk1. Fifty oocytes or embryos were collected in 2 μL Yamada's medium with 0.01 mM EDTA. Then, 10 μL of PBS supplemented with 2 mM PMSF, (2 times concentrated) were added to the samples followed immediately by 10 μL of SDS (2 times concentrated) and the samples were frozen in liquid nitrogen. After denaturing by boiling for 3 min, the proteins were aligned in polyacrylamide gel (11°C, 30 min, 60 V) (SDS-PAGE) and then separated on a 15 cm long 12.5% SDS-PAGE resolving gel (2 h 45 min, 400 V) in order to obtain a good separation of the bands corresponding to the different states of phosphorylation of cdk1. Proteins were then transferred onto a Hybond ECL nitrocellulose membrane (Amersham, Gent, Belgium) with a Transblot Semi-Dry Electrophoretic Transfer Cell (BioRad, Nazareth, Belgium) for 40 minutes according to the manufacturer's instruction. The quality of the blotting was verified by the use of kaleidoscope prestained standards (BioRad, Nazareth, Belgium). The membrane was then rinsed in TBS-T buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 10 min, then incubated with blocking reagent (Amersham) for 1 hour, rinsed three times in TBS-T buffer and finally incubated with 1:20 dilution anti-Cdk1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membrane was then rinsed 4 times with TBS-T, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody diluted to 1:500 in TBS-T and washed 5 times in TBS-T. The antibody adsorbed was located on the membrane by use of an ECL Western Blotting Detection System (Amersham, UK) according to the manufacturer's instructions and the membrane was then exposed to a sheet of autoradiographic film. The mouse RSV-3T3 fibroblast cell line expressing the active cdk1 was used as positive control (Transduction Laboratories, Lexington, KY, USA).

Statistical analysis. The results of liquid scintillation counting were expressed as the mean in cpm ± standard error of the mean (SEM). Student's *t*-test analysis was used for statistical evaluation of the results. Differences were considered to be statistically significant (as shown by *) or highly significant (as shown by **) if the *p*-value was less than 0.05 or 0.001, respectively.

Results

Changes of histone H1 kinase activity and the state of phosphorylation of cdk1 in control oocytes and early embryos. Studies performed in many different eukaryotic cells have shown that during interphase, cdk1 associates with cyclin and the formation of this complex allows for the phosphorylation of cdk1 on the Y15 and T14 residues (Figures 1 and 2A). Activation of cdk1 requires its dephosphorylation, which occurs just before mitosis. At the end of mitosis, cdk1 becomes inactivated by the destruction of cyclin (Figures 1 and 2A).

To be able to compare data in control conditions, a first set of experiments was performed on single BALB/c, Heiligenberger, C57BL and CF1 MII oocytes and one-cell embryos. The results confirmed that levels of histone H1 kinase activity could be estimated even in single cells (16, 18-20). The mean values of histone H1 kinase activity found in single oocytes and embryos collected during the early developmental stages of the four mouse strains are represented in Figure 2B. A high level of histone H1 kinase activity could be detected in MII oocytes of all strains. Low levels of activity were found immediately after fertilization, in early pronuclear embryos. Thereafter, histone H1 kinase activity rose to high values during mitosis of the first embryonic division, declined to basal levels at the early 2-cell stage and rose again at metaphase of the second division, in all investigated strains. The levels of activity found in MII oocytes or embryos in metaphase of the first or second mitosis were significantly higher than those found in embryos at the pronuclear stage or at the early 2-cell stage ($p<0.001$). Interestingly, the values obtained for embryos in the first mitosis were also significantly higher than those obtained for MII oocytes ($p<0.001$).

Differences in histone H1 kinase activity must normally be reflected by modifications in the state of phosphorylation of cdk1. This was determined in BALB/c and C57BL embryos, using western blotting techniques. This study was not performed in the Heiligenberger and CF1 strains, due to the difficulties in obtaining high numbers of embryos (50 for each single group) in these strains.

According to earlier studies in mouse oocytes and one-cell embryos (21), cdk1 would show three different migrating bands (referred to as upper, middle and lower bands) after electrophoretic separation and immunoblotting with an anti-cdk1 antibody. The upper and middle bands would be the phosphorylated (and therefore inactive) forms of the protein, since these two bands shift to the lower one with alkaline phosphatase treatment. We found that in MII oocytes, only the dephosphorylated active form of cdk1, as

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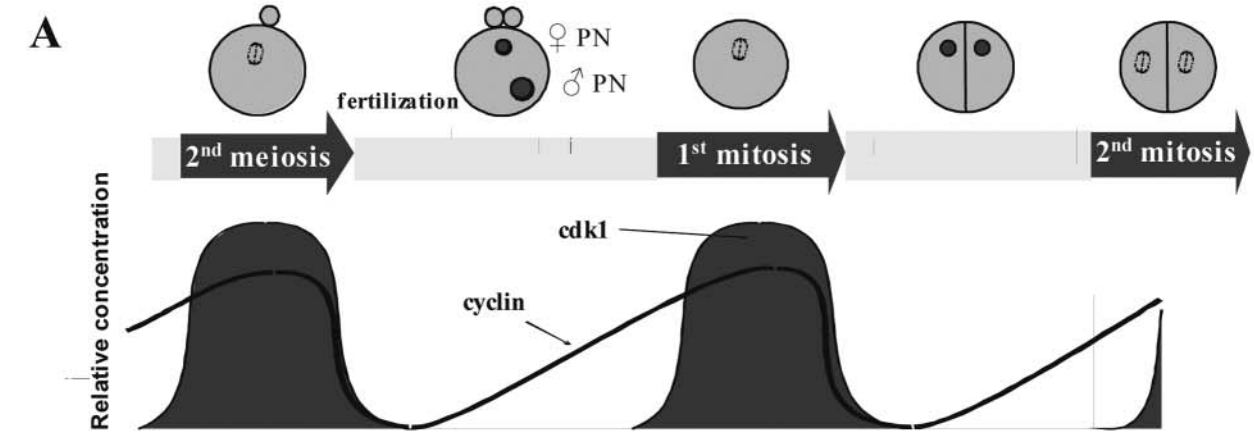
Figure 2. *(A) Cyclic kinase activity in oocytes and early embryos. (B) Comparison of histone H1 kinase activities in BALB/c, C57 BL, CF1 and Heiligenberger MII oocytes, 1-cell embryos at the pronuclear stage or in mitosis and 2-cell embryos at the mid-2 cell stage or in mitosis (mean activity ± SEM). (C) Phosphorylation state of cdk1 in oocytes (GV and MII) and embryos (1st mitosis, mid-2-cell stage). 3T3 cells were used as a positive control (active cdk1 resulting in one band). The lower band representing the tyrosine/threonine dephosphorylated form of cdk1 is seen in the positive control, in the MII oocytes and in the 1-cell embryos in 1st mitosis. The inactive form of cdk1 (as seen in GV oocytes and in embryos of mid-2-cell stage) is represented by three bands: the uppermost band is the phosphorylated form, the middle band is a transient form with only one amino acid phosphorylated, the lower band is the dephosphorylated form of cdk1.*


A
♀ PN
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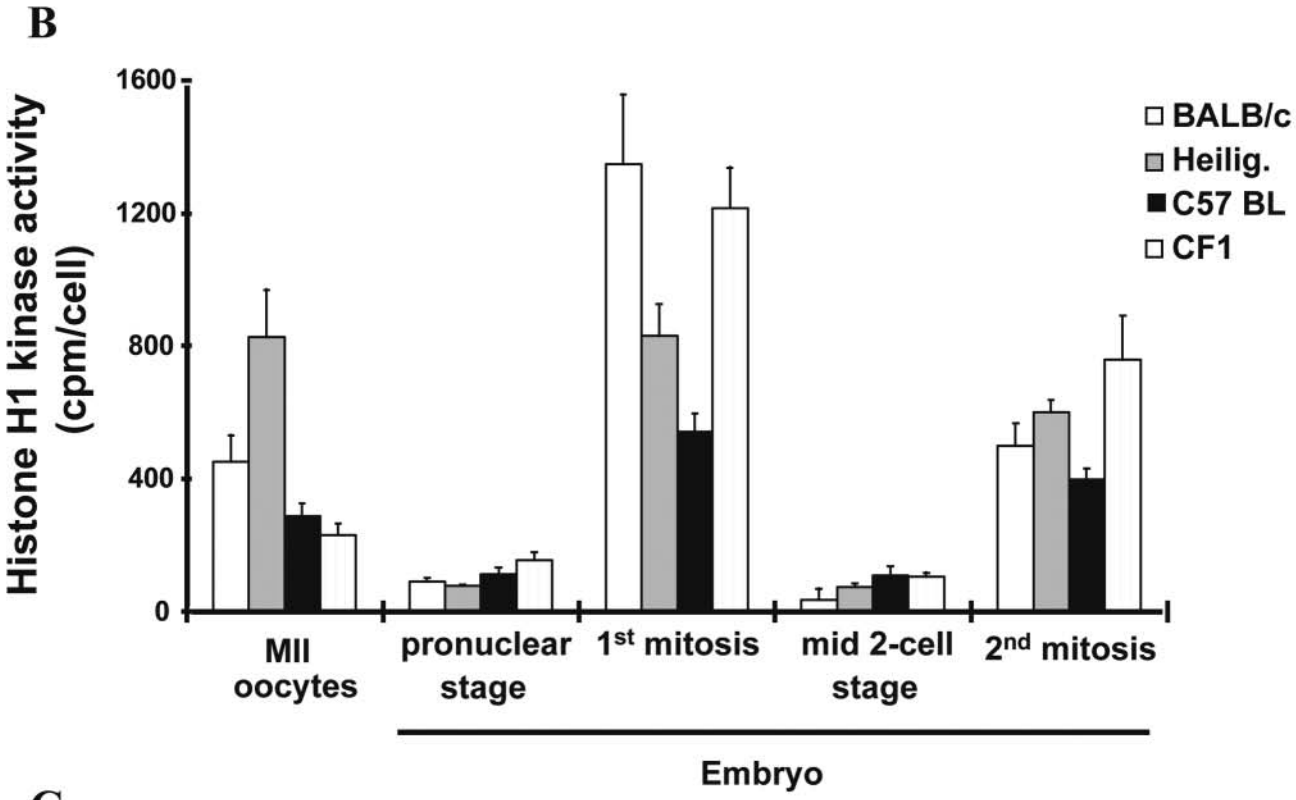
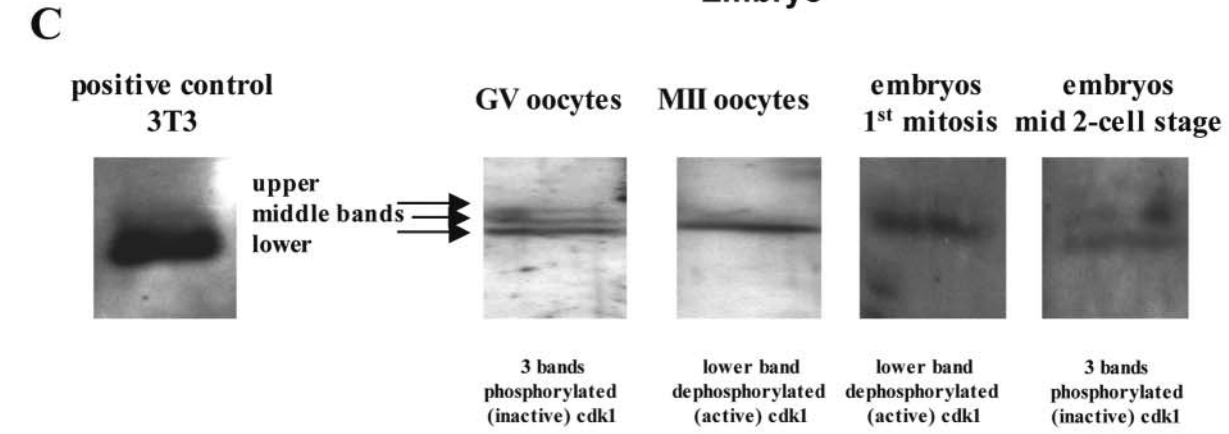
fertilization
2nd meiosis
1st mitosis
2nd mitosis
Relative concentration
cdk1
cyclin


B
Histone H1 kinase activity (cpm/cell)
1600
1200
800
400
0
BALB/c
Heilig.
C57 BL
CF1
MII oocytes
pronuclear stage
1st mitosis
mid 2-cell stage
2nd mitosis
Embryo


C
positive control 3T3
upper
middle bands
lower
GV oocytes
MII oocytes
embryos 1st mitosis
embryos mid 2-cell stage
3 bands phosphorylated (inactive) cdk1
lower band dephosphorylated (active) cdk1
lower band dephosphorylated (active) cdk1
3 bands phosphorylated (inactive) cdk1

evidenced by the presence of only one band in the autoradiograms, was present (Figure 2C). Just after fertilization, the amount of the dephosphorylated form decreased, whereas that of the phosphorylated form increased, as evidenced by the presence of three bands. During the first embryonic mitosis, cdk1 was again essentially represented by its lower band, and a similar situation prevailed during the second mitosis. These results were, therefore, in good agreement with those obtained in the histone H1 kinase assay.

Effects of X-irradiation on histone H1 kinase activity and the state of phosphorylation of cdk1 in 1-cell embryos. Histone H1 kinase activity was then investigated in X-irradiated one-cell embryos of the different strains. Embryos of BALB/c and Heiligenberger strains revealed great sensitivity to the radiation-induced G2-arrest, although Heiligenberger embryos reacted to irradiation in a more 'classic' way than BALB/c embryos, in that *all* irradiated embryos suffered a G2-arrest whose duration was proportional to the dose delivered. In BALB/c embryos, an 'all-or-none' effect was observed and the duration of the G2-arrest in blocked embryos was apparently not affected by the dose of radiation given.

A dose of 2.5 Gy was first administered to the pregnant Heiligenberger females 8 h *p.c.*, *i.e.* in early S-phase of the first embryonic cell-cycle (Figure 3A). Surprisingly, this dose exerted even more effects on Heiligenberger embryos than on BALB/c ones, in that nearly all irradiated embryos remained blocked at the one-cell stage. However, one embryo was able to divide after a 'normal' G2-arrest of a few hours. This embryo showed similar values of histone H1 kinase activity as control embryos in the first mitosis, while embryos unable to recover from G2 arrest showed significantly lower values (Figure 3A). When the dose of radiation was reduced to 0.5 Gy, embryos suffered an apparently short G2-arrest which was, however, difficult to evaluate due to the fact that even in the control group, there was always some asynchrony in the time of first division. The histone H1 kinase activity found in dividing irradiated embryos was not diminished (Figure 3B). In contrast, activity found during the slight G2-arrest was significantly lower than those found in dividing embryos. A dose of 1 Gy was chosen, which induced a G2 arrest of 3-4 h in all embryos. The values of histone H1 kinase activity found in irradiated embryos in delayed mitosis were not significantly different than those found in control embryos, while they remained at a very low level during G2 arrest (Figure 3C). The dose of 1 Gy was finally chosen for all experiments concerning the irradiation of the 1 and 2-cell mouse embryos of the Heiligenberger strain. The dose of irradiation for the three other mouse strains was 2.5 Gy.

Histone H1 kinase activities of 1-cell embryos of the four mouse strains were then evaluated after X-irradiation during the S-phase of the 1-cell embryo. The histone H1 kinase activities remained at a very low level during G2-arrest (Figure 4A). Heiligenberger embryos dividing after G2-arrest showed slight activity; though not at significantly lower values than those of the dividing controls. On the contrary, the values found in BALB/c embryos dividing after G2-arrest were very low, and hardly higher than those found in embryos that remained permanently blocked. In contrast, in C57BL and CF1 embryos dividing after a short G2-arrest the level of histone H1 kinase activity was high, and quite comparable, although not significantly higher, to that found in dividing control embryos.

In a next step, the state of phosphorylation of cdk1 was followed in irradiated one-cell embryos (Figure 4B). In the BALB/c strain, a shift to the upper and middle bands was noticed during G2-arrest, corresponding to the phosphorylated forms of cdk1. A low proportion of BALB/c embryos was able to escape the G2-arrest (5 to 10% after 2.5 Gy) and divided during the normal period of first mitosis, like control embryos. For these embryos collected at mitosis, only the lower band of cdk1 was visible, like in dividing control embryos. Forty to 50% of the irradiated G2-arrested embryos divided after a delay of nearly 20 h. For these embryos too, only the lower and active band of cdk1 was visible, although histone H1 kinase activity had been shown to remain very low in such embryos.

Likewise, in the C57BL strain, control and irradiated one-cell embryos reaching mitosis did not show any difference in the state of phosphorylation of cdk1: they only displayed the lower band of cdk1, corresponding to its active form (data not shown).

Effects of X-irradiation on histone H1 kinase activity and state of phosphorylation of cdk1 in 2-cell embryos of three mouse strains. Histone H1 kinase activity was measured and compared in two-cell embryos of the BALB/c, Heiligenberger and C57BL mouse strains (Figure 5A). For

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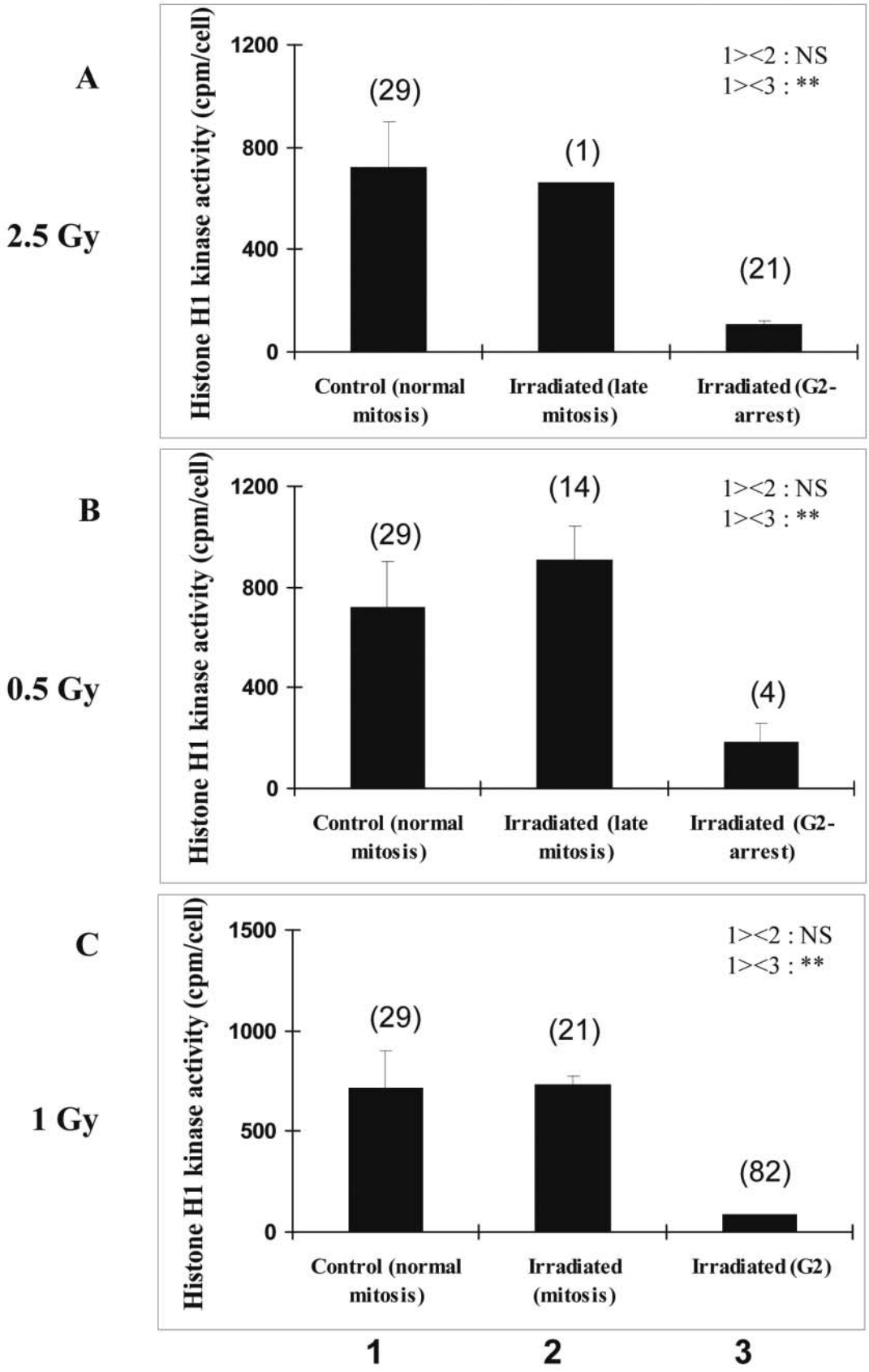
Figure 3. *(A) Histone H1 kinase activity in control and X-irradiated (2.5 Gy) 1-cell embryos of the Heiligenberger strain (mean activity ± SEM). NS: not significant, **p<0.001. After 2.5 Gy, all but one embryo remained blocked in the G2-phase of the one-cell stage. Therefore, a lower dose of 0.5 Gy was tested. (B) Histone H1 kinase activity in control and X-irradiated (0.5 Gy) 1-cell embryos of the Heiligenberger strain (mean activity ± SEM). When irradiated with a 0.5 Gy dose, almost none of the 1-cell embryos blocked in G2 and proceeded straight to mitosis. Therefore, it was decided to irradiate with a higher dose (1 Gy). (C) Histone H1 kinase activity in control and X-irradiated (1 Gy) 1-cell embryos of the Heiligenberger strain (mean activity ± SEM). With this dose, around half of the embryos blocked in G2 whilst the other half did not block and proceeded to mitosis.*


A
2.5 Gy
Histone H1 kinase activity (cpm/cell)
1200
800
400
0
(29)
(1)
(21)
1><2 : NS
1><3 : **
Control (normal mitosis)
Irradiated (late mitosis)
Irradiated (G2-arrest)
B
0.5 Gy
Histone H1 kinase activity (cpm/cell)
1200
800
400
0
(29)
(14)
(4)
1><2 : NS
1><3 : **
Control (normal mitosis)
Irradiated (late mitosis)
Irradiated (G2-arrest)
C
1 Gy
Histone H1 kinase activity (cpm/cell)
1500
1000
500
0
(29)
(21)
(82)
1><2 : NS
1><3 : **
Control (normal mitosis)
Irradiated (mitosis)
Irradiated (G2)
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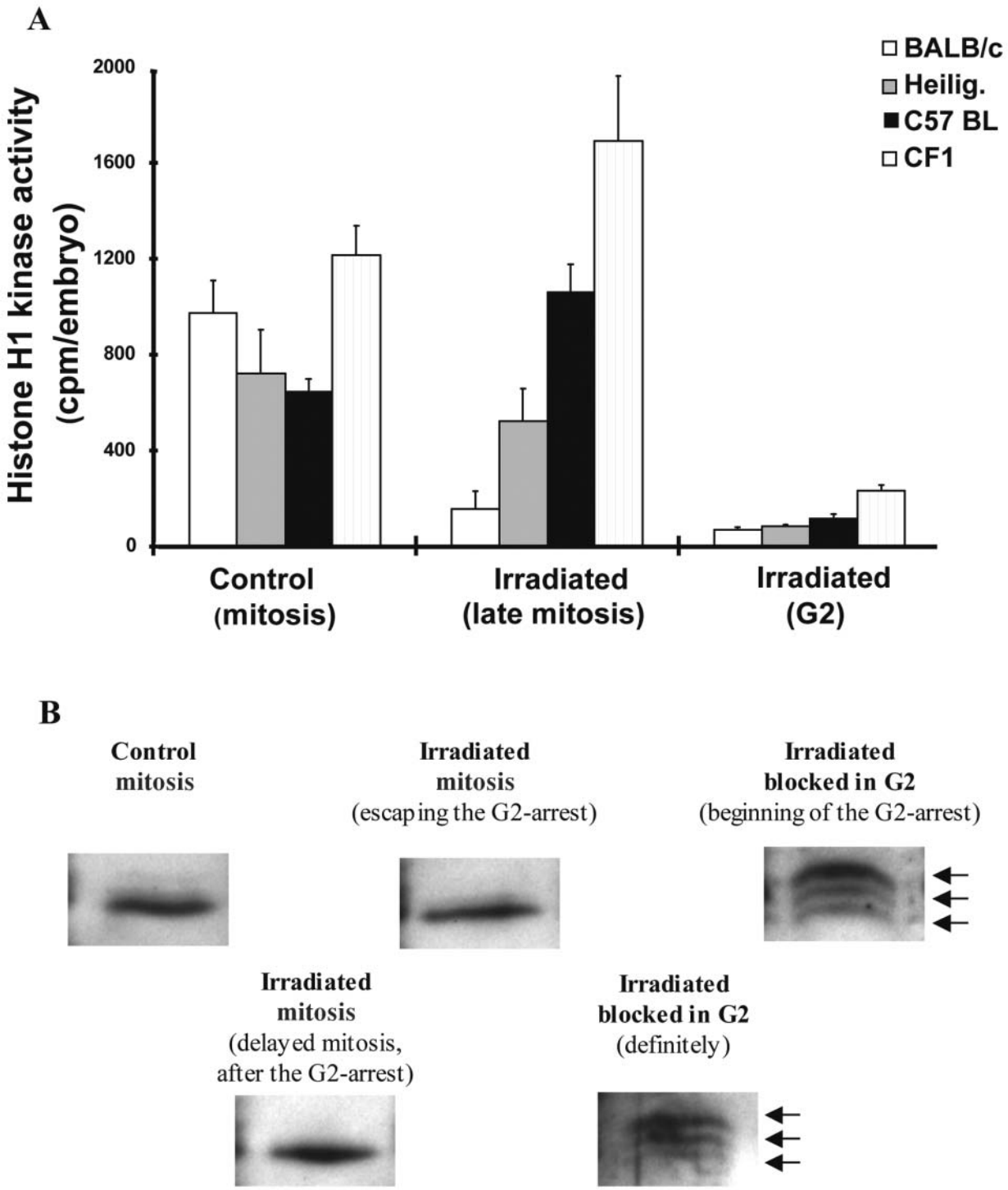

Figure 4. *(A) Comparison of histone H1 kinase activities in control and irradiated 1-cell embryos of the BALB/c, C57BL, Heiligenberger and CF1 mouse strains. BALB/c, C57BL and CF1 embryos were irradiated with 2.5 Gy whilst Heiligenberger embryos were irradiated with 1 Gy (B) Representative blots of cdk1 kinase activity in control and irradiated 1-cell embryos of the BALB/c mouse strain. The active form of cdk1 as represented by the lower band (corresponding to the phosphorylated form of cdk1) is seen in 1-cell embryos in mitosis: in control conditions, in irradiated embryos having escaped the G2-arrest and having reached mitosis, and in irradiated embryos having blocked in G2 for a few hours and having then reached mitosis. The inactive form of cdk1 is represented by three bands (as seen in 1-cell embryos blocked at the beginning of the G2 or definitively blocked in G2): the upper most band is the phosphorylated form, the middle band is a transient form with only one amino acid phosphorylated, the lower band is the dephosphorylated form of cdk1.*

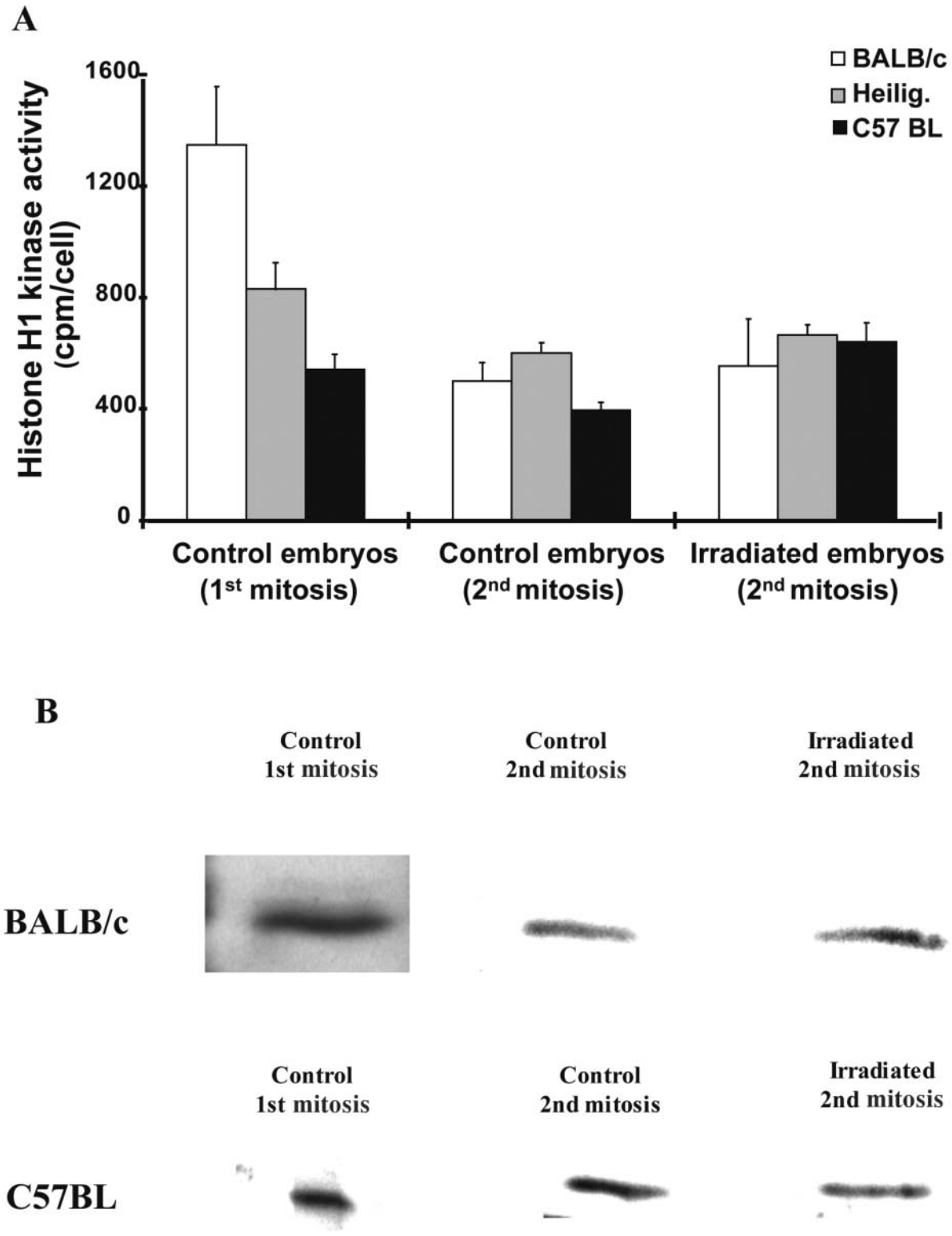

Figure 5. *(A) Comparison of histone H1 kinase activity in control and irradiated 2-cell embryos of the BALB/c, C57BL and Heiligenberger mouse strains. BALB/c and C57BL embryos are irradiated with 2.5 Gy, whilst Heiligenberger embryos are irradiated with 1 Gy. (B) Representative blots of cdk1 kinase activity in control and irradiated 2-cell embryos of the BALB/c and C57BL mouse strains. The active form of cdk1 as represented by the lower band (corresponding to the phosphorylated form of cdk1) is seen in control and irradiated 2-cell embryos in mitosis.*

that purpose, the embryos were irradiated in early G2-phase of the second cell-cycle. The G2-arrest induced in the two-cell embryos was extremely short in all three mouse strains. Histone H1 kinase activities were measured when two-cell embryos reached the second mitosis. Similar levels of activity were found in irradiated embryos of the three strains (Figure 5A). These were high and similar to the values found in dividing control embryos.

The state of phosphorylation of cdk1 was also investigated in two-cell embryos of the BALB/c and C57BL strains, after irradiation during early G2 phase of the second cell-cycle (Figure 5B). Embryos reaching mitosis after a short G2-arrest showed no difference in the state of phosphorylation of cdk1, with the appearance of only the lower band, indicating active cdk1.

Discussion

Exposure of mammalian cells to damaging agents can result in transient cell-cycle arrest or in apoptotic cell death. Although the processes of cell death and cell proliferation appear to be opposing and mutually contradictory, substantial evidence indicates that the two processes are linked (22-24). Transitions between different cell-cycle phases are regulated by surveillance mechanisms or checkpoints that monitor the integrity of the DNA. Cyclin-dependent kinase (Cdk) complexes, essential for cell-cycle transitions, are controlled by checkpoints and inappropriate cdk activity during cell-cycle transitions often correlates with apoptosis. For example, in some systems induction of apoptosis by various stimuli requires the activation of either cdk1 or cdk2 (25-29), whereas forced expression of cdk inhibitors prevents apoptosis in various cell types (29-32).

Histone H1 kinase was first identified in Chinese hamster cells as an enzyme whose activity increases during metaphase and that phosphorylates histone H1 in a cAMP-independent manner (33, see reviews in 34 and 35). High histone H1 kinase activities have been correlated to nuclear lamina disassembly (36), nucleolar disassembly (37), chromosome condensation (38), transcription regulation (39), translation regulation (40), microfilament rearrangement (41) and reorganization of the intermediate filaments (42). In yeast, marine vertebrates, amphibians and mammals, histone H1 kinase activity parallels that of the cdk1/cyclin A and cdk1/cyclin B complexes both required for entry into mitosis. Therefore, histone H1 kinase activity is widely used as a biochemical indicator of the kinase activity of the cdk1.

The first step of this work consisted in determining the modifications of histone H1 kinase activity occurring during the very early development of the mouse embryo of four mouse strains differing in their radiation sensitivities (BALB/c and Heiligenberger: radiation-sensitive; C57BL and CF1: radiation-resistant), using a sensitive method devised by Fulka *et al.* (16) which allows the determination of this activity in single cells. In agreement with the earlier work of these authors and of Jung *et al.* (41), we found that, in the four mouse strains, histone H1 kinase activity was high in ovulated oocytes in MII and that this activity strongly declined following fertilization and formation of the pronuclei. The activity increased sharply during the first mitotic division of the embryos, fell to basal values at the early 2-cell stage and increased again during the second mitotic division. The modifications of histone H1 kinase activity found during the one-cell and two-cell stages also confirm the results obtained in other mouse strains by Choi *et al.* (21) and by Aoki *et al.* (43) with methods involving lysates containing 50 or 10 oocytes/embryos, respectively.

Mouse embryos provide an excellent model to clarify the regulation of cdk1 activation during the G2/M transition since one can use the blocked embryos as a kind of 'mutant' and compare these embryos to those obtained from nonblocked strains. It is known that, in the mouse embryo, the development is regulated exclusively at a post-transcriptional level until the mid 2-cell stage, where the first RNA synthesis is detected (44, 45). One-cell embryos of some mouse strains are particularly sensitive to radiation-induced G2 arrest. The sensitivity of the embryo to this effect is determined by the maternal genotype and, of all studied strains, Heiligenberger embryos were found to be the most radiation-sensitive. Additionally, the peculiar radiation sensitivity of the embryo to the radiation-induced G2-arrest seems to be limited to the one-cell stage, since irradiation at later stages (two- or four-cell stages) revealed much less effect (results not shown). Since, in the mouse, transcription begins at the two-cell stage, there seems to be a strong link between the sensitivity to G2 arrest and the transcriptional inactivity/activity of the embryo. Results obtained in irradiated one-cell embryos indicated that in all mouse strains, histone H1 kinase activity did not exceed basal levels during the period of G2-arrest. However, irradiated embryos dividing after G2-arrest showed high levels of histone H1 kinase activity (except for the BALB/c strain). Furthermore, some strain-to-strain differences were noticed at the end of G2-arrest: i) Irradiated BALB/c embryos dividing after G2-arrest showed very low levels of histone H1 kinase activity. In fact, the values found in those late-dividing embryos were only slightly higher than those found in irradiated embryos that remained blocked in the G2-phase. The reason for these differences remains to be defined. However, earlier experiments performed in our laboratory conclusively showed that BALB/c embryos dividing after G2-arrest die very soon thereafter (data not shown). Thus, abnormally low levels of histone H1 kinase activity during mitosis could be indicative of some remaining damage, which would be expressed later as embryonic death, and the lower the level of activity, the

more rapid the embryonic death after division; ii) In irradiated Heiligenberger embryos dividing after G2-arrest, the values of histone H1 kinase activity were slightly, though not significantly lower than those found in dividing control embryos; iii) In contrast, irradiated C57BL and CF1 embryos dividing after G2-arrest showed very high levels of histone H1 kinase activity.

Embryos irradiated in the first cell-cycle were arrested during G2, however, the characteristic of this radiation-induced G2-block induced in the Heiligenberger and the BALB/c strains in the first embryonic cell-cycle is totally in between the two mouse strains tested. Heiligenberger embryos irradiated with increasing doses of X-rays developed a G2-block which was dependent in length on the radiation dose. This correlation between duration of the G2 block and the radiation dose is known from somatic cells. However, one-cell mouse embryos of the BALB/c strain exhibit a G2-block fixed in duration, which exactly fits into the time when control embryos start to progress into the next cell-cycle, *i.e.* irradiated and blocked BALB/c one-cell embryos skip one cell-cycle. The radiation dose in BALB/c embryos had no influence on the duration of the block, rather it determined the proportion of embryos which exhibited a cell-cycle arrest. The higher the dose, the higher the number of embryos that were blocked. This differential dynamics of the G2-block in one-cell embryos of the Heiligenberger and the BALB/c strain is paralleled by a similar dynamics in histone H1 kinase activity in these embryos. In G2-blocked embryos the activity was low; at the end of the block when the embryos entered the next mitosis the activity was high again (except in the BALB/c mouse strain). The peculiar sensitivity of the Heiligenberger and BALB/c one-cell embryo towards the radiation-induced G2-arrest most probably results from a deficiency or a lack of expression of components normally accumulated in the cytoplasm of the oocyte during oogenesis. However, irradiation of Heiligenberger and BALB/c 2-cell embryos does not result in a drastic G2-arrest (46), and this could be explained by the fact that transcriptionally active embryos are able to synthesize the lacking components. It could be reasonable to assume that a lack of repair enzyme(s) for some types of DNA damage could be partially or totally responsible for the peculiar G2-arrest observed in these one-cell embryos.

Conclusion

Cell-cycle effects in early embryos under normal conditions and after irradiation are paralleled by changes in the activity of the central cell-cycle driving enzyme complex, the cyclin B1/cdk1 complex. We have shown that after irradiation of the one- or two-cell embryos, this complex is inactivated and the cell-cycle progression is blocked. Resumption of cell proliferation is preceded by the activation of the complex.

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