Cell cycle-dependent regulation of the DNA-dependent protein kinase

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Abstract. Human DNA-dependent protein kinase (DNA-PK) is a nuclear-localized serine/threonine protein kinase. The holoenzyme consists of a catalytic subunit with a molecular mass of 465 kDa and a DNA-binding heterodimer Ku86/70. The kinase has been implicated in a variety of nuclear processes including V(D)J recombination, double-strand break repair, and transcription. Cells with defective DNA-PK activity show increased radiosensitivity and lack of V(D)J recombination. To study DNA-PK activity during the cell cycle, HeLa cells were separated by elutriation centrifugation into different cell cycle compartments based on cellular size. DNA-PK activity was found to vary during the cell cycle. The kinase activity was lowest during G1 phase and increased dramatically as the cells entered S phase and remained high during the G2-phase. The subcellular distribution of DNA-PKcs is relocated from the cytoplasm during M and G1 phases to the nucleus during G1–S phase transition and S phase. Expression of both the catalytic subunit and the Ku86/70 heterodimer was found to be constant throughout the cell cycle. This study demonstrates that DNA-PK activity as well as its subcellular localization fluctuates during the cell cycle. In addition, the distribution of DNA-PK during M phase corresponds with low DNA-PK activity.

INTRODUCTION

DNA damage caused by a broad range of agents, including radiation and mutagenic chemicals, may interfere in a deleterious way with both replication and transcription of the genome. When cells are X-irradiated, several types of DNA lesion are introduced. In growing cells this may result in delayed cell cycle progression and/or cell death. Several proteins may identify specific DNA lesions, monitor and signal incomplete and damaged DNA, ensuring that the DNA is repaired before the cell enters mitosis. The DNA-dependent protein kinase designated DNA-PK is involved in detecting and evoking an early response to DNA double-strand breaks (double-strand breaks) (Lees-Miller & Anderson 1989).

DNA-PKcs is a serine/threonine protein kinase with a molecular mass of 465 kDa. This kinase shows sequence homologies to PI-3 kinase-related proteins such as ATM, Rad3, Mec-1, TEL 1, TOR, FRAP; several of which are suggested to be involved in DNA-damage detection and repair (Keith & Schreiber 1995). For its activity, DNA-PKcs is strictly dependent upon Ku, a heterodimer consisting of two polypeptides of 70 and 86 kDa (Gottlieb & Jackson 1993, Suwa *et*

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al. 1994). DNA-PKcs is activated when Ku binds mainly to linear double-stranded DNA fragments but other DNA constructs including closed stem-loop structure and single stranded gaps can also effectively activate the kinase (Morozov *et al.* 1994).

Mutant cell lines defective in either component of DNA-PK show increased sensitivity to ionizing radiation. The cell lines derived from the immune-deficient scid mouse and the mutant rodent cell lines xrs-6 lack DNA-PKcs and Ku 86, respectively. These cell lines are defective in DNA double-strand break repair and V(D)J recombination, in addition to being hypersensitive to ionizing radiation (Gottlieb & Jackson 1993, Smider *et al.* 1994, Taccioli *et al.* 1994, Blunt *et al.* 1995, Kirchgessner *et al.* 1995, Lees-Miller *et al.* 1995). V(D)J recombination events have been shown to be restricted to the G1 phase of the cell cycle (Schlissel *et al.* 1993). The human RAD51 homologue (HsRAD51) protein involved also in repair and recombination has been shown to exhibit cell cycle-dependent expression with one peak in late S phase and one in late G2 (Flygare, Benson & Hellgren 1996). This observation suggests that DNA-PK being involved in recombination events also could be regulated in a cell cycle-dependent manner.

This paper demonstrates that the activity and subcellular localization of DNA-PK in HeLa cells is not constant during the cell cycle. The DNA-PK kinase in HeLa cells shows an increasing kinase activity from G1 to S phase. Western blot analyses show a constant expression of DNA-PKcs and Ku during all stages of the cell cycle. It is suggested that the relocation of the enzyme from cytoplasm to the nucleus is a process which involves activation of the protein kinase.

MATERIALS AND METHODS

Cell culture

HeLa cells were maintained in CO₂-independent medium (GIBCO BRL, Paisley UK) supplemented with 10% foetal calf serum and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin) at 37°C. Cells were harvested when subconfluent using 0.05% trypsin in phosphate-buffered saline (PBS).

Separation of cells

The cells were washed with Tris-buffered-saline (TBS; Tris 2 mM, NaCl 150 mM, pH 8.0), and separated according to cell cycle phase based on their cell size. Cell separation was carried out using a Beckman JE-G elutriation rotor with a Beckman J21 B centrifuge. The rotor speed was maintained at 950 *g* at 4°C. Fractions 1, 2, 3, 4, 5, and 6 were collected at buffer flow rates of 17, 26, 33, 48, 62 and 80 ml/min, respectively. Fraction one, containing mostly dead cells, was discarded. Fraction 6 was harvested after the rotorhead was stopped and the separation chamber cleaned by a maximum buffer rate of 80 ml/min. The relative numbers of cells in the various phases of the cell cycle were determined by quantitative DNA analysis using flow cytometry (PASII; Portec, Münster, Germany).

Cell extracts

Separated cells were washed with TBS and lysed by freezing and thawing once in a volume equivalent to three-times the cell pellet volume with low salt buffer (10 mM HEPES pH 7.6, 25 mM KCl, 10 mM NaCl, 1.1 mM Mg₂Cl, 0.1 mM EDTA, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 10 µg/ml TPCK) and incubation on ice for 20 min. The samples were centrifuged for 10 min at 100 000 *g*. Supernatants were collected and the protein concentrations determined. Recovered supernatants were stored at -80°C.

Cell synchronization

HeLa cells were kept at log phase. To synchronize cells in G₁, cells were incubated with hydroxyurea for 24 h at a concentration of 1 mM. Subsequently cells were washed with PBS and resuspended in PBS for cytospin and kinase activity. To arrest cells in mitosis, cells were treated with nocodazole for 24 h at a concentration of 100 ng/ml. Preparation of cell extracts was performed as above.

Immunofluorescence

HeLa cells were cytospun, fixed with 4% formaldehyde and incubated overnight at 4°C with anti-DNA-PKcs (AHP 318) as a primary antibody. An anti-rabbit Fab fragment conjugated with fluorescein isothiocyanate (FITC) was used to visualize the DNA-PKcs. The nuclei and chromosomes were labelled with DAPI fluorescence stain.

Determination of DNA-PK kinase activity

Samples containing 25 µg protein were assayed for DNA-PK activity by a DNA-PK assay kit (Amersham, Amersham, UK). Briefly, extracts were mixed with a specific substrate peptide (a part of the p53 peptide that is phosphorylated by DNA-PK) contained in the DNA-PK assay kit, ds-DNA and ATP in a HEPES buffer (10 mM HEPES pH 7.2, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA). Assay mixtures contained 11.1 MBq [³³P]ATP per assay. As a negative control assay mixtures without ds-DNA were used. The reaction mixtures were incubated at 37°C for 20 min. The reactions were stopped with the stop buffer included in the kit and spotted onto Whatman P81 filter papers. The filters were washed twice for 2 min in 25 mM H₃PO₄ and three times for 2 min in Milli-Q purified water.

Immunoblots

Extracts adjusted to an equivalent amount of protein (60 µg) were loaded on 4–15% linear gradient SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto a nitrocellulose filter by the semi-dry method (Bio-Rad Trans-Blot; Sundbyberg, Sweden). Western blots experiments were performed as described by the manufacturer's instructions (ECL; Amersham). The specific proteins were detected by rabbit polyclonal anti-DNA-PKcs (AHP 318; Serotec, Oxford, UK), anti-Ku 70 and 86 (AHP 316, AHP 317 respectively; Serotec) at a 1 : 5000 dilution. The proteins were visualized with an ECL Western blot detection kit (Amersham).

RESULTS

Cell cycle distribution

Figure 1 shows fractions from a representative elutriation. Analysis of the DNA content of cell populations isolated by elutriation showed that different fractions contain cells belonging to G₁ (upper left), an accumulation in S phase (upper right), and an accumulation in G₂ (lower left). Cells arrested at the G₁–S border by hydroxyurea (lower right) and cells arrested in M by nocodazol (bottom).

DNA-PK activity

As illustrated in Figure 2a, DNA-PK activity could be detected in all cell fractions. However, cells in fractions enriched for cells in G₁, show an activity 80% lower than that in fractions enriched for cells in S phase. Cells in G₂ contained 88% of the activity in S phase cells but as

Figure 1. DNA analysis of separated HeLa cells. This shows the DNA analysis of representative elutriation. Top left; cells enriched in G1. Top right; cells enriched in S. Lower left; cells enriched in G2. Lower right; cells arrested in G1S border by hydroxyurea. Bottom; cells arrested in M phase by nocodazol treatment.

Figure 2. The DNA-PK specific activity in HeLa cells from different cell cycle fractions. The results are expressed as the mean of normalized kinase activity values from four different experiments. G1 represents elutriation fractions containing 84–88% G1 cells, S represents elutriation fractions containing 10–48% S phase cells, and G2 represents elutriation fractions containing 31–44% G2 cells. Inset; DNA-PK activity in HeLa cells treated with hydroxyurea and nocodazol. The activity is expressed in arbitrary units. Kinase activity was determined as described in *Materials and methods*.

can be seen (Figure 2), the standard deviation is quite large as a result of different accumulations of G2 cells in different experiments. Cells with the highest content of cells in G2 showed slightly lower kinase activity than cells with the highest percentage of cells in S phase. Thus, the lowest kinase activity was found in the cell cycle fraction containing 88% G1 cells and maximum activity was found in fractions with the highest content of S phase cells. To measure the DNA-PK activity in cells arrested in G1–S phase and mitosis, cells were treated with hydroxyurea and nocodazol, respectively. Cells arrested in G1–S transit showed approximately 50% higher activity than cells arrested in mitosis after treatment with nocodazol (Figure 2 inset). These results suggest a decreased kinase activity during mitosis compared to late G1 and S phase. Cells not yet arrested in M phase might have contributed to the activity observed in the nocodazol treated cells.

Determination of DNA-PK content

To see if the observed differences in DNA-PK activity between G1, S and G2 phase cells corresponded to a similar alteration in the expression of the holoenzyme, the content of DNA-PKcs and Ku 70 and 86, respectively, were measured in cells of different cell cycle

Figure 3. The content of DNA-PKcs, Ku (80), and Ku (70). (a) In HeLa cells. Lane 1 corresponds to cells enriched in G1 phase (84–88% enrichment); Lane 2 to cells enriched in S phase 40–44% enrichment); Lane 3 to cells enriched in (G2 31–44% enrichment). The content of the DNA-PKcs and Ku components were determined by Western blotting as described in *Materials and methods*.

compartments. The different fractions obtained were analysed by Western blot analysis. As shown by Figure 3 all fractions, G1, S, and G2, displayed the 465 kDa DNA-PKcs product and Ku 86/70 antigens. In addition, the amounts of these components did not seem to differ between the cell cycle fractions (Figure 3). These observations exclude the possibility that the fluctuations in DNA-PK activity during the cell cycle are caused by alterations in the content of the enzyme components.

Subcellular distribution of DNA-PKcs

As DNA-PK is a nucleic localized protein kinase, it was of interest whether there was a cell cycle dependent relocalization of the protein kinase that could explain the fluctuations in kinase activity during the cell cycle. In the indirect immunofluorescent experiment, AHP 318 was used as primary antibody along with Fab fragments conjugated with green FITC label. Although the DNA-PKcs was mainly localized to the nucleus during late G1 and S (Figure 4), DNA-PKcs was found to some extent in the cytoplasm during all cell cycle phases. This is in agreement with earlier observations on extracts from cytoplasm and nuclei (Carter *et al.* 1990; Danska *et al.* 1996). However, by measuring the presence of DNA-PKcs in cells accumulated in different cell cycle phases, an accumulation of the protein in the nucleus was noted during the late G1 and S + G2 phases (Figures 4a,f). Figure 4e,f suggests that DNA-PKcs is localized in the cytoplasm and not in the area surrounding the condensed chromatin during mitosis. To further confirm the observation that DNA-PKcs is localized in the cytoplasm during M phase as well as that DNA-PKcs is accumulated in the nucleus during late G1, cells were arrested at the G1–S border using hydroxyurea and in M phase using nocodazol. As seen in Figure 4g–j DNA-PKcs is poorly represented in the area surrounding

Figure 4. Subcellular localization of DNA-PKcs. (a,c,e,g,i) Nuclei (DAPI), (b,d,f,h,j) DNA-PKcs (FITC) cells enriched in G1 phase (a,b), cells enriched in S phase (c,d), cells enriched in G2-phase (e,f), HeLa cells arrested in G1-S border by hydroxyurea (g,h), and HeLa cells arrested in M phase by nocodazol (i,j). As described in *Materials and methods*.

the condensed chromatin during mitosis (Figure 4i,j). The protein kinase accumulates in the nucleus during G1-S transition (Figure 4g,h).

DISCUSSION

The DNA-dependent protein kinase is thought to function as a central component of the DNA damage surveillance system. The present study shows that DNA-PKcs and Ku86/70 are present in all cell cycle phases of HeLa cells. This is in accordance with previously published data from HL-60 and HeLa cells (Yaneva & Jhiang 1991, Ajay *et al.* 1995, Lee *et al.* 1997). The lower DNA-PK activity observed in G1 cells as compared to S and G2 cells may reflect an important role for DNA-PK during the latter phases. It is possible that, as S and G2 cells contain more DNA, the activity of a DNA-damage surveillance protein such as DNA-PK would be increased during these phases. The activity is not regulated by the expression of the different subunits that comprise in the DNA-PK enzyme. An interesting observation is that the 34 kDa subunit of RPA which is a substrate for DNA-PK is hyperphosphorylated during S and G2 phases (Din *et al.* 1990). In addition, radiation-induced RPA hyperphosphorylation is defective in scid mutations (Boubnov & Weaver 1995). Thus, the high DNA-PK activity during S and G2 phases, demonstrated in the present study, coincides with the hyperphosphorylation of the DNA-PK substrate RPA.

While this work was in progress, Lee *et al.* (1997) published evidence for cell cycle-dependent DNA-PK activity. By using nocodazol and hydroxyurea synchronized cells Lee *et al.* show a cell cycle-dependent DNA-PK activity and sensitivity to X-irradiation. The present study confirms the cell cycle-dependent regulation of DNA-PK. By using elutriated cells and thereby omitting the interference of drugs, DNA-PK activity, but not its content, was shown to be changing during the cell cycle. However, no decrease was seen in kinase activity during S phase or an additional increase in kinase activity during G2 phase as reported by Lee *et al.* The reason for the observed differences in DNA-PK kinase activity in S and early G2 cells is not obvious but differences in cell preparation and synchronization may be contributing factors. The G2 fractions in the present study contain both G1 and S phase cells, and S phase cell fractions could be contaminated with late G1 early S phase cells. However, the G1 phase fractions showing the lowest DNA-PK activity contain over 80% G1 cells and fractions containing cells enriched in S and G2 show high DNA-PK activity. In addition, there was a 50% decrease in kinase activity during M phase compared to G1-S phase cells when the cells were arrested with nocodazol and hydroxyurea, respectively. The activity that was still measurable in nocodazol-treated cells could be emanating from cells not in mitosis and/or from a low activity in the mitotic cells. G1 phase cells express the lowest DNA-PK activity during the cell cycle and the activity increases at the G1-S transit. The kinase activity remains at the same level throughout S and G2 phase followed by a decrease during mitosis.

By the use of immunohistochemistry, a cell cycle-dependent relocalization of the enzyme could be seen. It is suggested that in late G1 and S phase, DNA-PKcs is localized to the nucleoplasm. During mitosis, DNA-PKcs could not be observed in the same area as the condensed chromatin. A translocation of DNA-PKcs has also been suggested after growth stimulation of T-lymphocytes (Nagasawa *et al.* 1997). They showed an increased DNA-PKcs activity and protein content in the nuclei upon stimulation of proliferation, i.e. cell populations going from resting to driving through the cell cycle with an increased fraction of S phase cells.

Metzger & Iliakis 1991 concluded from their observations that there are no cell cycle-dependent fluctuations in the ability to repair DNA double-strand breaks in CHO cells. If the DNA-PK activity directly corresponds to DNA-PK dependent double-strand break repair, their results are in variance with those from the present study. However, since DNA-PK phosphorylates a multitude of *in vitro* substrates, suggesting an involvement in many nuclear processes, it can not be ruled out that the higher DNA-PK activity in the S phase as compared to G1 reflects a DNA-PK-dependent process not necessarily directly linked to DNA double-strand break repair. More likely, DNA-PK activity reflects different mechanisms of illegitimate repair of DNA double-strand break during different cell cycle phases, which is suggested by Lee *et al.* (1997).

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