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p53-Independent checkpoint controls in a plant cell model

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ABSTRACT

Allium cepa L. meristems were used as a plant model to study the p53-independent control of S and G2 phases by checkpoint pathways, in eukaryotic cells. Checkpoint blocks were induced at early and mid S by hydroxyurea. After their spontaneous override, cells became accumulated in G2-prophase, giving rise later on to a delayed mitotic wave. Cell growth was maintained during the checkpoint blocks, as the delayed mitoses were larger in size than the control ones. Under continuous hydroxyurea treatment, the delayed mitotic was formed by two subpopulations: normal mitoses corresponding to cells having properly recovered from the checkpoint block, and abnormal ones resulting from checkpoint adaptation. These latter cells displayed broken chromatids as they had unduly overriden the G2 checkpoint block, without completing DNA repair. The frequency of the checkpoint-adapted mitoses increased with the hydroxyurea concentration from 0.25 to 1.0 mM. However, from 1 mM hydroxyurea upwards, some of the cells lost their competence for checkpoint adaptation. Therefore, the dose of a genotoxic agent that still allows G2 checkpoint adaptation should always be applied in order to get rid of uncontrolled proliferating cells. This is specially suitable for cells lacking a functional p53 protein.

Key terms: Checkpoint adaptation; Hydroxyurea; S and G2 checkpoints; p53-independent checkpoints; Allium cepa L. meristems.

INTRODUCTION

Most of the checkpoint proteins responsible for the transduction of antimitogenic signals which prevent an irreversible cycle phase transition, before some crucial requirement is fulfilled, are conserved in eukaryotes. Thus, the codifying gene for the kinase mutated in patients with ataxia telangiectasia (ATM) is also present in plants (García et al., 2000). This kinase activates the Cdc25 phosphatase that, in turn, activates the cyclin-dependent kinases (CDKs), by removing the inhibitory phosphorylations in the CDK threonine 14 -tyrosine15 residues. These residues behave as a molecular switch which alternatively activates or brakes cycle progression. There is no Cdc25 sequence homologue in plants. However, some functional homologue of this phosphatase should be present in plants, as dephosphorylation of the CDK inhibitory phosphoresidues also takes place in response to cytokinin activation (Reichheld et al., 1999).

The universality of the genes that stimulate proliferation (Cdc genes, proto-oncogenes) and also those that brake it (Chk or checkpoint genes, tumor suppresor genes) justify the use of different eukaryotic model systems to deal with still uncertain features of the proliferation control.

Sequential waves of different CDKs complexed with different regulatory cyclins promote cycle progression throughout the different cycle phase transitions (Pines and Rieder, 2001). In plants, the functional homologue of the animal Cdk1 is CdkA, while there are two CdkB1 and CdkB2

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groups not present in animal cells (Joubès et al., 2000). In relation to cyclins, plants possess three A-type and two B-type cyclin groups (Renaudin et al., 1998). Expression of both B-type cyclins are restricted to late G2 and mitosis (Hirt et al., 1992; Fobert et al., 1994). Plant cyclin B2 seems to be the homologue of the mammalian cyclin A. During G2, it is expressed before cyclin B1. Cyclin B2 seems to be the target of the checkpoint control pathways operating in G2 and early mitosis (Weingartner et al., 2003). The ectopic expression of cyclin B2 induces the override of the G2 checkpoint that surveys the irreversible entry into mitosis when activated by topoisomerase II inhibition (Giménez-Abián et al., 2002).

Root meristems can be used to study the behaviour of the eukaryotic proliferating cells. They keep a convenient proliferation fraction (close to 1.0), and maintain all the checkpoints intact, in contrast to immortalized cells. Moreover, as the checkpoint or tumor suppressor protein p53 has not been detected in plants (Arabidopsis Genome Initiative, 2000; Vandepoele et al., 2002), the plant cells here used can be considered as a suitable model for studying the p53-independent control of proliferation. This is also the case in nearly 50% of tumoral mammalian cells (López-Saéz et al., 1998).

In the present work, we have followed S checkpoints activated by hydroxyurea and the subsequent G2 checkpoint in *Allium cepa* L. plant meristems. The depletion that this drug induces in deoxyribonucleotides, by blocking the ribonucleotide reductase (Graslund, 2002), challenges the intra-S or replication checkpoints and, also, the G2 checkpoint in sequence (Pelayo et al., 2001).

The checkpoint pathways transduce antimitogenic signals that provide the proliferating cell with additional time to accomplish a necessary requirement before the irreversible transition to a subsequent cycle phase. Thus, they prevent G2 cells entering mitosis until the completion of DNA replication and post-replication repair are completed (Melo and Toczyski, 2002; Samuel et al., 2002).

The arrest in cycle progression provided by a checkpoint is always transient, either due to cell recovery (fulfilment of the requirement) or to checkpoint adaptation. In this latter process, the cell spontaneously continues cycle progression despite unfulfilment of the checkpoint requirement. Checkpoint adaptation is often followed by genome instability, allowing cell survival at the cost of tolerating mutation.

The fates of both the recovered and the checkpoint-adapted cells were followed during the continuous presence of different HU concentrations. Cells lost their capability to recover and become checkpoint-adapted when overriding a certain treatment concentration threshold.

MATERIAL AND METHODS

Bulbs of *Allium cepa* L. cv. Francesa were obtained in the local market. The bulb scales were removed and, after washing the bulbs, their crowns where root blastema are located were placed on 90-ml glass tubes filled with filtered tap water. The water was continuously aerated by bubbling air at 10-20 ml/min. They were kept in dark growth incubators at 25 ± 0.5 °C. The filtered tap water was renewed every 24 h, while drug treatments were changed at 8 or 12 h intervals. Two or three days after initiating the cultures, bulbs with more than 40 roots were selected. Each bulb was cut in two halves, and the cut surfaces were extensively washed in filtered tap water. Before any treatment was initiated, each half-bulb was again cultured for at least 8 h under control conditions. Each half-bulb was placed on a new culture tube containing either the control or the other experimental solutions. Hydroxyurea (Sigma) solutions were freshly prepared in filtered tap water previously warmed to 25 °C.

Isolation of nuclei

Nuclei were isolated as described by Lucretti and Dolezel (1995) with minor modifications. Briefly, one millimeter long root segments corresponding to 0.5 to 1.5

mm from the root tip were fixed for 20 min at 4 °C in 4% (v/v) formaldehyde in Tris buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 7.5) containing 0.1% Triton X-100. After fixation, samples were washed three times with Tris buffer. Segments from the meristems were homogenised in lysis buffer (15 mM Tris, 2 mM EDTA, 80 mM KCl, 20 mM NaCl and 0.1 Triton X-100, pH 7.5), using a dispersing homogeneiser (Ultra Turrax T20, 25N-8, IKA, Germany). After mincing, homogenates were filtered through a 50 µm stainless-steal mesh. The nuclear suspensions obtained were centrifuged at 600 g for 20 min at 4 °C. Then supernatants were removed and the pellets were resuspended in the lysis buffer and maintained at 4 °C until the flow cytometry analyses were performed. At each fixation time, a total number of 10,000 nuclei were studied by flow cytometry.

Isolation of cells and recording of cell size

In order to record cell size by flow cytometry, whole cells were isolated from root segments previously fixed for one hour. The protocol for isolation of nuclei was followed. However, after washing with the Tris buffer, 10 mM citrate buffer pH 4.8 was added for cell isolation. The Tris buffer was again used 3 times, before adding the lysis buffer at pH 4.8. Cell size was evaluated by recording light scatter values by flow cytometry. At the same time, the relative amount of DNA was estimated, after propidium iodide (PI) staining of nuclei. These values were used to estimate the cell size to nuclear DNA content ratios.

Flow cytometry

DNA content was estimated after PI staining and flow cytometry analysis. Samples were incubated with RNase A (Boehringer-Mannheim) at 30 µg/ml (equivalent to 1.5 i.u. per sample), and propidium iodide (Sigma) at 100 µg/ml, for 30 min at room temperature. Flow cytometry analysis was carried out with an EPICs XL analyzer (Coulter, Dunstable, UK), equipped with an ion argon laser tuned at 488 nm. Fluorescent signals from the PI-labelled nuclei were collected by a 525-nm BP filter and a 620-nm BP filter (both from Zeiss), respectively. For DNA content analysis, doublets and larger aggregates were discarded by electronic analysis of integral- and height-signals from the particles analyzed.

Conventional optical microscopy

Mitotic index and the frequency of metaphases and telophases with and without chromatid breaks were recorded in root tips fixed overnight in 3:1 (v/v) ethanol-acetic acid, and then stained with acetic orcein. An average of 6,000 cells were scored at each treatment, from at least three roots of different bulbs. The ratio of normal to aberrant anaphases-telophases was also estimated.

RESULTS

The effect of hydroxyurea on DNA replication and on cell size

The cell distribution in relation to DNA content in the cells that were kept in the continuous presence of 0.75 mM HU (treatment renewed every 8 h to exclude HU breakdown) is presented in Figure 1. It shows that DNA synthesis was reinitiated within 10 h of continuous HU treatment, when some cells reached a bulk DNA content close to 3C. There is a second replication checkpoint in this mid S position, where cells accumulate again (Pelayo et al., 2001).

The cell size (light scattering) and nuclear DNA contents (propidium iodide signal) obtained by flow cytometry were used to estimate the cell size to DNA content ratio in meristems treated with hydroxyurea for 14 h (equivalent to one cell cycle time in control conditions), as well as in control meristems. The cell size to DNA content ratios were similar in the non-replicating 2C cells of HU-treated and untreated meristems (Fig. 2). However, the cells with

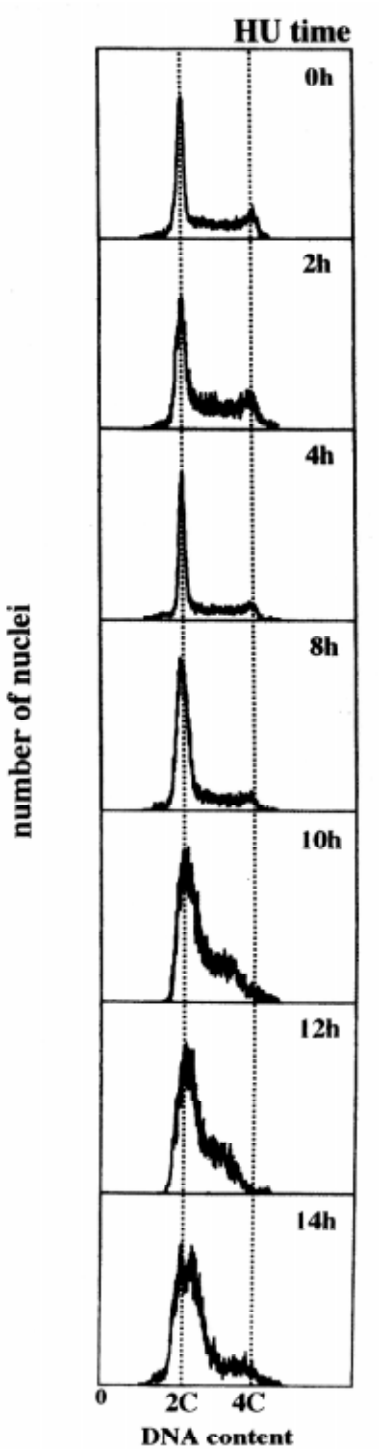

Fig. 1. Cell distribution at different times of 0.75 mM HU treatment in relation to DNA content, assessed by the propidium iodide incorporated by their nuclei. Asymmetry between both slopes of the 2C peak becomes detectable at the hour 10. The checkpoint override of the HU-induced early S block starts to occur in some cells of the population at that time, when cells with a DNA content around 3C accumulate. At the 14th h, the cell accumulation in mid S is also overriden.

DNA values between 2 and 4C displayed a greater size to DNA content ratio in the HU-treated cells than in the controls. This means that the cells accumulated in the S phase in the presence of HU endured continuous cell growth while their DNA replication was checkpoint-blocked.

The delayed appearance of a mitotic wave

The frequency of mitoses with and without broken chromatids (abnormal and normal mitoses, respectively) were scored in cells in the continuous presence of 0.75 mM HU. HU produced a continuous fall in the mitotic index (MI) up to the 10 h of treatment, when the MI was closest to zero (Fig. 3). MI started to rise again mostly from the 28 h onwards of the continuous HU treatment. The frequency of the normal mitoses in the 36 to 42 h of hydroxyurea reached a value higher than the normal frequency of mitotic cells found in controls, at the 0 h.

From 16 to 34 h of HU treatment, most of the mitotic cells were abnormal, i.e. they displayed chromosomes with chromatid breaks. This observation directly proves that these latter cells were adapted to the G2 DNA damage checkpoint. On the other hand, in the interval between 36 to 42 h, the frequency of normal mitoses (with apparently intact chromosomes) rose over the frequency of the abnormal ones. This increment in normal mitoses corresponds to cells that have recovered from the HU-induced chromosomal damage during either the replication or G2 checkpoint-induced cycle blocks. It is surprising that such a recovery could take place in the continuous presence of HU.

Effect of HU concentration on the frequency of G2 checkpoint-adapted cells in the delayed mitotic wave

In order to determine whether there is any effect of the HU concentration on the chromosomal damage in the abnormal mitotic cells, the frequency of mitoses with chromosomal aberrations was estimated, under six different HU concentrations (0.25

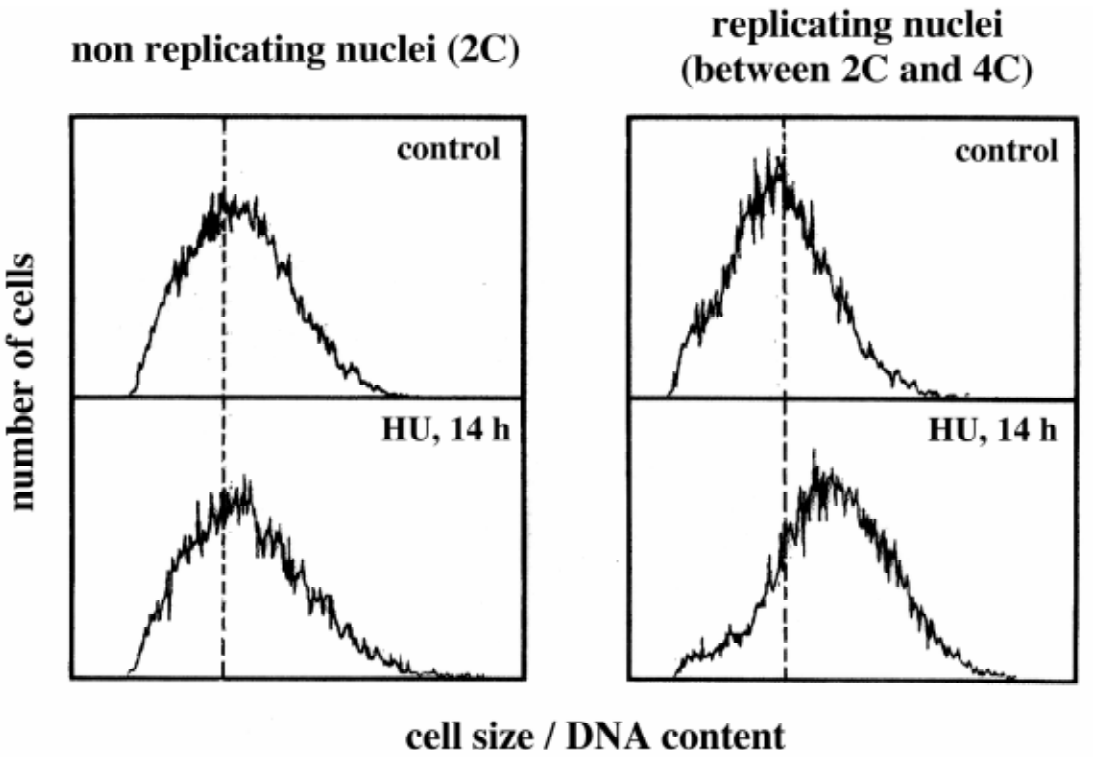

Fig. 2. Comparison of cell distribution at different cell size to nuclear DNA content ratios, in HU-treated and untreated cells. Left panels show the values corresponding to non-replicating G0 and G1 cells. Those corresponding to replicating cells are in the rigth panels. The discontinuous vertical lines indicate the mean values recorded in the controls.

to 1.5 mM), at 26, 32 and 38 h of continuous HU treatment. At these three treatment times, there was a positive linear correlation (r = 0.9995; 0.9885 and 0.990, respectively) between the frequency of mitotic cells with chromosomal aberrations and the HU concentration, between 0.25 and 1.0 mM (Table I). The maximum frequency of mitoses with broken chromatids (98.3%) was recorded at 1.0 mM HU, 26 h after the HU treatment had been initiated. The delay in appearing such a late mitotic wave was independent from the HU concentration, at least in the 0.25 to 1.5 mM range (data not shown).

At all concentrations assayed, the frequency of mitoses with broken chromatids fell progressively down from the 26 to the 38 h of recovery. Strikingly, the abnormal to normal mitoses ratio decreased with the dose in the 1.25 and 1.5 mM HU treatments, when compared to the values obtained at 1.0 mM HU (Table I). However, the frequency of normal mitoses remained steady when compared to those found at 1 mM HU concentration (data not shown). Therefore, some of the cells that should have entered into the delayed wave of mitoses lost their competence for checkpoint-adaptation over a certain damage threshold.

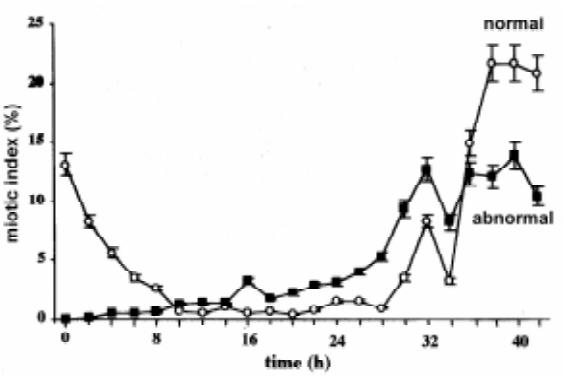

Fig. 3. Mitotic index for normal (open circles) and abnormal mitoses (closed squares) recorded under 0.75 mM HU. The presence of chromatid breaks in the abnormal mitoses indicates that these cells have endured adaptation at least to the DNA damage checkpoint in G2 and, probably, to the replication checkpoints. For any time point, the total mitotic index corresponds to the sum of the frequencies of both normal and abnormal mitoses. From the 16 to 34 h of HU treatment, the frequency of abnormal mitoses was higher than that of normal ones. The trend was reversed from later times (36 to 40 h).

TABLE I

Frequency of the checkpoint-adapted mitoses (i.e. those with chromosomal aberrations) at different HU concentrations (mean ± SD)

Treatment duration, h	HU concentration, mM					
	0.25	0.50	0.75	1.0	1.25	1.5
26	26.1 ± 2.3	48.3 ± 4.0	72.9 ± 6.4	98.3 ± 8.6	63.0 ± 5.1	20.1 ± 1.8
32	20.3 ± 4.1	31.8 ± 2.4	60.2 ± 5.3	78.6 ± 6.4	56.4 ± 4.7	16.2 ± 2.8
38	12.9 ± 1.2	26.1 ± 1.9	36.4 ± 3.5	43.2 ± 3.8	29.6 ± 3.2	8.7 ± 1.3

DISCUSSION

Redundant checkpoint pathways control common cycle phase transitions both in yeast (De la Torre-Ruiz et al., 1998), in human (Passalaris et al., 1999) and in plant cells (Pelayo et al., 2001). These latter cells provide a valid model for studying the p53-independent control of G2 that could also apply to 50% of the mammalian transformed cells that lack a functional p53 checkpoint protein.

All checkpoint pathways incorporate different inputs to give rise later to a single mitogenic/antimitogenic output. Cell size is one of such checkpoint inputs. An enlarged size provides an excess of mitogenic signals. Cell enlargement promotes the override of the pheromone-induced G1 checkpoint block that makes *S. pombe* mating partners competent for conjugation (Stern and Nurse, 1997). The inputs on excessive cell size may not be restricted to G1 and G2 phases. The enlargement recorded in the plant cells that resumed DNA replication in the presence of HU supports that it also applies to the intra-S checkpoints. As a result of the checkpoint operation, there would be a cross-talk between the mitogenic signals, produced by continuing cell growth, and the antimitogenic ones, produced by the replication forks that are stalled in the HU presence.

Adaptation to intra-S checkpoint blocks can be possible in the continous presence of HU if the nucleotide pool rise is due to the upregulation of the ribonucleotide reductase genes (Elledge et al., 1993).

Cell subpopulation recovered from checkpoint blocks

The additional time needed by proliferating cells to accomplish any missing task before a phase transition, that is provided by a transient checkpoint block, can be called acclimation time. This term is borrowed from the adaptive response to environmental stress. The present results show that cells transiently blocked by an intra S checkpoint activated by HU are able to complete both their DNA synthesis and their post-replication repair. These cells enter into mitosis with all their chromosomes apparently intact, and then are indistinguishable from controls (Pelayo et al., 2001). Such cell subpopulation apparently recovers from the stress produced by nucleotide depletion which is the main objective of the replication checkpoint pathway (Desany et al., 1998).

Checkpoint-adapted cells

The proper HU recovery response is different from checkpoint adaptation. Instauration of alternative unconventional routes (Kohn, 1999) permits the fulfilment of the missing requirement. Checkpoint override before fulfilment of a requirement offers the cell the option to remain viable at the expense of its genome instability (McClintock, 1984).

Cells unable to perform checkpoint adaptation may instead induce a program of cell death (apoptosis), or may simply fail to proliferate, remaining quiescent. Re-

initiation of proliferation in them is responsible for tumor growth relapse.The present data suggests that the choice of alternative outcomes depends on the stress intensity, a function of the type and amount of cell damage. Thus, over a certain threshold, the frequency of cells unable to become checkpoint-adapted increased with the HU concentration.

The induction of checkpoint-adaptation potentiates the damaging effect of the genotoxic agents used in cancer therapy (Ghosh et al., 1997). As this work shows, the concentration of the genotoxic agent determines the amount of cells that endure checkpoint adaptation. Therefore, the determination of the maximum concentration of a genotoxic treatment that still allows checkpoint adaptation is needed to optimize it in order to get rid of transformed cells.

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