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Influence of the G_2 cell cycle block abrogator pentoxifylline on the expression and subcellular location of cyclin B1 and $p34^{cdc2}$ in HeLa cervical carcinoma cells

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Abstract. The progression of cells from G_2 into mitosis is mainly controlled by formation of the cyclin B1/$p34^{cdc2}$ complex. The behaviour of this complex in the irradiation-induced G_2 cell cycle delay is still unclear. A prior study demonstrated that the expression of the cyclin B1 protein is reduced by irradiation, and restored to control levels by the methylxanthine drug pentoxifylline, which is a potent G_2 block abrogator. The present study shows that irradiation, and 2 mM pentoxifylline affect the expression of the cyclin-dependent kinase $p34^{cdc2}$ in HeLa cells. Irradiation induces $p34^{cdc2}$ levels to increase and cyclin B1 levels to decrease. Addition of pentoxifylline at the G_2 maximum reverses these trends. This is also evident from the cyclin B1/$p34^{cdc2}$ ratios which decline after irradiation and are rapidly restored to control levels upon addition of pentoxifylline. It is concluded that cyclin B1 and $p34^{cdc2}$ protein expression are important events and act in concert to control the irradiation induced G_2 block. Analysis of cyclin B1 expression in whole cells and in isolated nuclei furthermore show that cyclin B1 is translocated from the nucleus into the cytoplasm when the G_2 block is abrogated by pentoxifylline.

INTRODUCTION

G_2 arrest is a response exibited by proliferating eukaryotic cells exposed to a variety of DNA damaging agents including X-irradiation (Weinert & Hartwell 1988), DNA alkylators (Konopa 1988) and topoisomerase inhibitors (Barlogie *et al.* 1976). It is presumed that G_2 arrest facilitates DNA repair prior to mitosis. Exposure of irradiated cells to methylxanthines like caffeine and pentoxifylline induces mitosis before DNA repair is complete. This is thought to contribute to the enhanced cell killing observed when cells are irradiated in the presence of methylxanthines (Lau & Pardee 1982). The biochemical mechanisms underlying the formation of the G_2 block and its drug induced abrogation are not yet well understood.

Entry into mitosis is dependent on the activation of the maturation promoting factor (MPF) which contains a 34 kDa serine threonine cyclin-dependent protein kinase, $p34^{cdc2}$.

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Kinase activity is controlled by the formation of a complex between $p34^{cdc2}$ and the mitotic cyclin B1 protein (Murray *et al.* 1989; Broek *et al.* 1991). Upon cyclin B1 binding, $p34^{cdc2}$ becomes phosphorylated at threonine 161, which promotes affinity for cyclin B1, and at threonine 14 and tyrosine 15 by the Wee1 kinase, which inhibits kinase activity (McGowan & Russel 1993). The subsequent dephosphorylation of threonine 14 and tyrosine 15 residues by the cdc25 phosphatases activates $p34^{cdc2}$ kinase (Murray 1992). The activation is also regulated by the subcellular location of the $p34^{cdc2}$ complex (Li, Meyer & Donoghue 1997). The cyclin B1/$p34^{cdc2}$ complex, which is localized in the cytoplasm during interphase, is transported into the nucleus at the onset of mitosis (Pines & Hunter 1991) and then phosphorylates nuclear substrates, e.g. the condensin complex (Murray 1998)

It is clear that the formation and activation of the MPF complex is controlled at multiple levels. Recent investigations address the role of MPF in the formation of the G_2 cell cycle arrest in greater detail. It is now generally thought that control of entry into mitosis involves the expression of cyclin B1 (Muschel *et al.* 1991; Maity *et al.* 1996) and p34 ^{cdc2} (Lock & Ross 1990), the activation of the MPF complex by dephosphorylation (McGowan & Russel 1993) and subcellular translocation (Li *et al.* 1997).

Irradiation depresses cyclin B1 expression (Bernhard *et al.* 1994a; Kao *et al.* 1997; Hwang & Muschel 1998). Data from the authors' laboratory show that the addition of pentoxifylline to G_2 blocked cells induces cyclin B1 expression (Theron & Böhm 1998). Other authors have shown similar results in synchronized cells for caffeine and staurosporine (Bernhard, McKenna & Muschel 1994b). The aim of this study was to evaluate the role of $p34^{cdc2}$ protein expression in the radiation induced G_2 arrest, and the influence of the G_2 abrogator pentoxifylline. The importance of using asynchronous cell populations in this type of study has been previously emphasized (Theron & Böhm 1998), in order to avoid the unscheduled expression of cyclins (Gong, Traganos & Darzynkiewics 1995). $p34^{cdc2}$ expression has previously been measured by flow cytometry in polyploid cells (Baroja *et al.* 1996). To the authors' knowledge this aspect has not been investigated flow cytometrically in response to DNA damage and G_2 block abrogation. Some authors suggest that the levels of $p34^{cdc2}$ remain constant throughout the cell cycle and also after irradiation (Lock & Ross 1990; Lock 1992; McGowan & Russel 1993). The present flow cytometric results in HeLa cells allow the correlation of $p34^{cdc2}$ expression directly with cell cycle stage and showed a higher percentage of $p34^{cdc2}$ expression in the G_1 and G_2 phases, with an increase in $p34^{cdc2}$ expression in G_2 during the radiation induced G_2 block.

The measurement of cyclin B1 expression has previously lead to confusing reports on changes in protein expression. This can be attributed to the sharp rise in the numbers of G_2 cells during block formation which obscures the change in the relative proportions of cyclin B1 to G_2 cells. To avoid this pitfall, cyclin B1 expression was expressed as a ratio of cyclin B1 to the fraction of cells in G_2 (Theron & Böhm 1998). Accordingly, $p34^{cdc2}$ expression was measured by calculating $p34^{cdc2}/G_2$ ratios.

The activity of the maturation promoting factor also strongly relies on the subcellular location after the binding of the cyclin B1 and $p34^{cdc2}$ constituents (Moore *et al.* 1999). It has been suggested that the cyclin B1/$p34^{cdc2}$ complex is localized in the cytoplasm during interphase and transported into the nucleus at the onset of mitosis (Pines & Hunter 1991; Tassan *et al.* 1994). This was confirmed by using a fusion protein between cyclin B1 and green fluorescent protein to trace the movement of MPF in subcellular components (Hagting *et al.* 1998). Cyclin B1 has been reported to appear in the cytoplasm during late S phase, and then moves to the perinuclear region and during G_2 enters the nucleus, where it is concentrated during mitosis (Kakino *et al.* 1996). A unique cytoplasmic pool of cyclin B1 has

also been documented for actively dividing cells, which rapidly increases during S phase and G_2 phase and is then translocated to the nucleus during early prophase where it forms a complex with the nuclear subset of p34^{cdc2} (David-Pfeuty & Novain-Dooghe 1996). It is now generally thought that the inactive cyclin B1/p34 complex indeed shuttles between the nucleus and cytoplasm in human cells (Pines 1999).

This study used asynchronous HeLa cells and flow cytometry to examine the expression of p34^{cdc2} and the subcellular location of the MPF complex by analysing cyclin B1 expression in isolated nuclei and in whole cells after exposure to ionizing irradiation and after G_2 block abrogation. The results clarify molecular events which contribute to the formation of the radiation induced G_2 cell cycle delay and the mechanism of action of pentoxifylline as a G_2 block abrogator.

MATERIALS AND METHODS

Cell culture

Asynchronous populations of HeLa cervical carcinoma cells were plated in McCoy's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum in 75 cm^2 culture flasks and cultured at 37°C in 95% O_2/5% CO_2. Samples in exponential phase were irradiated with 7 Gy of ^{60}Co and allowed to grow for 10 h, which is the time needed for maximum G_2 block formation in HeLa cells. Pentoxifylline was added to specific flasks to a final concentration of 2 mM. At different time intervals thereafter, ranging from 2 to 25 h, the pentoxifylline treated and control samples were trypsinized, fixed in 70% ethanol and stored at –20°C.

Determination of G_2 block

Time of maximum cell cycle block in G_2 was determined by irradiating HeLa cells with 7 Gy of ^{60}Co, and sampling at 2-h intervals for up to 25 h. The cells were fixed in 70% ethanol and stored overnight at −20°C. After washing in phosphate buffered saline (PBS), cells were resuspended in PBS containing 10 μg propidium iodide (PI; Sigma, St Louis, MI, USA) and 0.1% RNase A. Samples were incubated at 37°C for 20 min prior to flow cytometric analysis.

Immunocytochemistry

Fixed cells were prepared for multiparameter flow cytometry to simultaneously measure total DNA content and cyclin B1 (Gong *et al.* 1994) or p34^{cdc2} expression (Baroja *et al.* 1996) as previously described.

Briefly, the cells were washed in PBS and treated with 0.25% Triton X-100 for 5 min on ice. After another wash in 5 ml PBS, the cell suspension of 5×10^5 cells/100 μl was incubated overnight at 4°C in a 1:400 dilution of mouse monoclonal anti-cyclin B1 antibody (Pharmingen Clone GNS-1, San Diego, CA, USA), or a purified monoclonal anti-p34^{cdc2} antibody (clone HCDC1, ICN Biochemicals) in PBS containing 1% bovine serum albumin (BSA). The next morning, cells were washed in PBS and incubated for 30 min at room temperature in a 1:40 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma) in PBS/1% BSA. The cells were washed again, resuspended in 10 μg/ml PI and 0.1% RNase A in PBS, and incubated for 20 min at room temperature prior to analysis. Negative controls were prepared in a similar way, except that an isotype-specific antibody, mouse IgG (Sigma), was used instead of the cyclin B1 or anti-p34^{cdc2} antibody.

Flow cytometry

To determine cyclin B1 or $p34^{cdc2}$ levels, samples were analysed on a Becton-Dickinson (San Jose, CA, USA) FACScan flow cytometer. Fluorescence data from 10 000 events were collected, stored and analysed using Lysis II software. To determine the time of maximum G_2 block, samples were analysed for red (PI) fluorescence which was displayed as a DNA histogram. Markers placed at the G_2 boundaries served to estimate the G_2 content for each time point.

Cyclin B1 or $p34^{cdc2}$ expression, and DNA data, were displayed in dot plots of red (PI) vs. green (FITC) fluorescence representing total cellular DNA content and cyclin B1 or $p34^{cdc2}$ expression, respectively. Cell doublets were gated out by using the doublet discrimination module. Determination of the G_2 content was as described above. The fraction of cells expressing either cyclin B1 or $p34^{cdc2}$ was determined by gating only cells that displayed a positive green (FITC) fluorescence. The threshold for FITC positive cells was defined using the gate window set on the negative control sample which was prepared with the isotype-specific antibody IgG1, on all the treated samples. All experiments were repeated at least twice and generated identical trends.

Definition of cyclin $B1/G_2$, $p34^{cdc2}/G_2$ and cyclin $B1/p34^{cdc2}$ ratios

The cyclin $B1/G_2$ and $p34^{cdc2}/G_2$ ratios were calculated by comparing the fraction of cells expressing either cyclin B1 or $p34^{cdc2}$ to the fraction of cells in the G_2 phase of the cell cycle for each post-irradiation time point. Cyclin $B1/p34^{cdc2}$ ratios were obtained by dividing the $B1/G_2$ ratios over the $p34^{cdc2}/G_2$ ratios.

Isolation of HeLa nuclei

Nuclei were obtained as described (Heussen *et al.* 1987). Briefly, cells from exponentially growing cultures were washed once in 'lysis' buffer consisting of 10 mM Tris–HCl, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.4. Cells were then resuspended in ice-cold lysis buffer at $1–3 \times 10^6$ cells/ml and allowed to swell for about 15–20 min on ice. Lysis of cells was completed by the dropwise addition of 10% (v/v) nonidet-P40 (Shell Chemical Co.) in lysis buffer to a final concentration of 0.5% (v/v). During this step, the sample was mixed vigorously on a vortex. Released nuclei were sedimented at 300 ***g*** for 5 min in a swing bucket rotor and resuspended gently by stepwise addition of small volumes of PBS, pH 7.4, with gentle mixing on a vortex, essential to prevent clumping. The isolation of nuclei was confirmed by microscopy, and the sample was then subjected to the normal staining procedure for cyclin B1.

RESULTS

In HeLa cells, the radiation-induced G_2 cell cycle block reaches a maximum at 10–12 h after a single dose of 7 Gy (Figure 1). The DNA histogram shows estimates of normal cell cycle fractions of 53% and 30% in G_1 and G_2, respectively. These values change to 30% and 62% for G_1 and G_2, respectively, at 10–12 h post-irradiation. Pentoxifylline was added at this estimated time for maximum G_2 block expression. From the dotplot of red (PI) vs. green (FITC) fluorescence it is evident that, unlike cyclin B1 (Theron & Böhm 1998), $p34^{cdc2}$ is not limited to the G_2 phase of the cell cycle, but is also expressed in G_1 phase cells (Figure 2a). However, it is predominantly expressed in the G_2 phase during G_2 arrest (Figure 2b).

Figure 3a shows the increase in G_2 fraction during radiation induced G_2 arrest and the abrogation of the G_2 block when pentoxifylline is added at the time of maximum G_2 block.

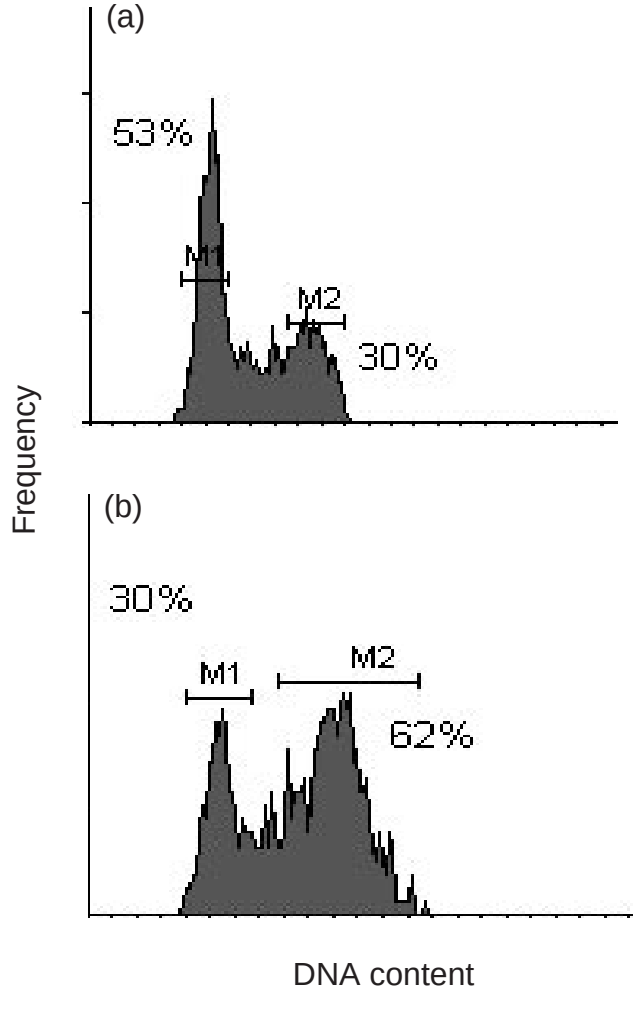

Figure 1. Flow cytometric histogram of the total DNA content of HeLa cells, indicating the normal cell cycle distribution (a) and the formation of a G_2 block at 12 h post-irradiation (b).

Similarly the expression of p34^{cdc2} increases after irradiation and drops after pentoxifylline treatment (Figure 3b). When the ratios of p34^{cdc2} to G_2 fraction are plotted over time, an overexpression of p34^{cdc2} during G_2 arrest becomes evident. Pentoxifylline treatment reduces the p34^{cdc2} expression to the control level of 0.1 (Figure 3c). Figure 4 shows the cyclin B1/G_2 ratios for similar dose and time points in HeLa. Since cyclin B1 and p34^{cdc2} act in concert, the cyclin B1/p34^{cdc2} ratio was also plotted (see definition in *Materials and methods* section). Figure 5 shows that the G_2 block maximum is associated with a very low cyclin B1/p34^{cdc2} ratio which gradually increases as the cells recover from the G_2 block and enter mitosis after 40 h. Pentoxifylline added at the maximum G_2 block rapidly restores the cyclin B1/p34^{cdc2} ratio over a very narrow time window, which reaches the control value after ~7 h. Abrogation of the G_2 block thus is associated with a rapid restoration of the critical cyclin B1/p34^{cdc2} ratio.

In order to study the influence of pentoxifylline on subcellular translocation of the cyclin/cdk complex, cyclin B1 expression was compared in isolated nuclei and whole cells. Addition

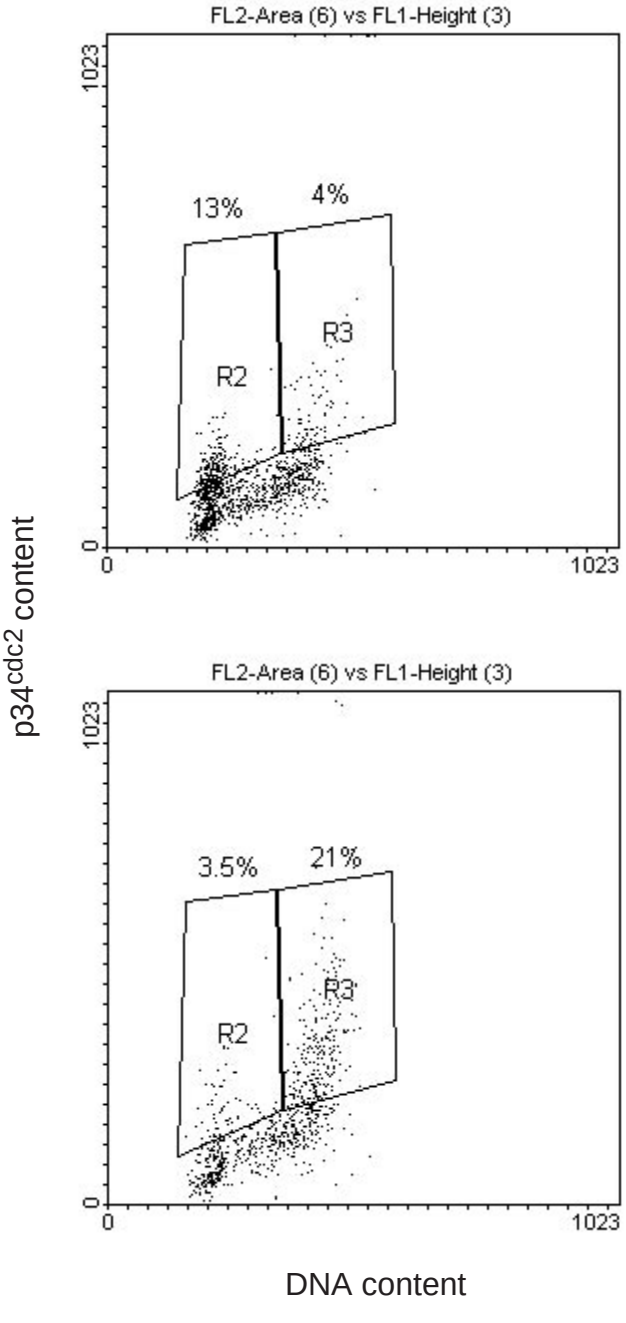

Figure 2. Dot plots of green fluorescence (FITC) on the vertical scale vs. red fluorescence (PI) on the horizontal scale, representing $p34^{cdc2}$ content and total DNA content, respectively. Percentages of cells expressing $p34^{cdc2}$ are indicated for HeLa cells in the G_1 and G_2 phases, before (a) and 12 h after irradiation (b).

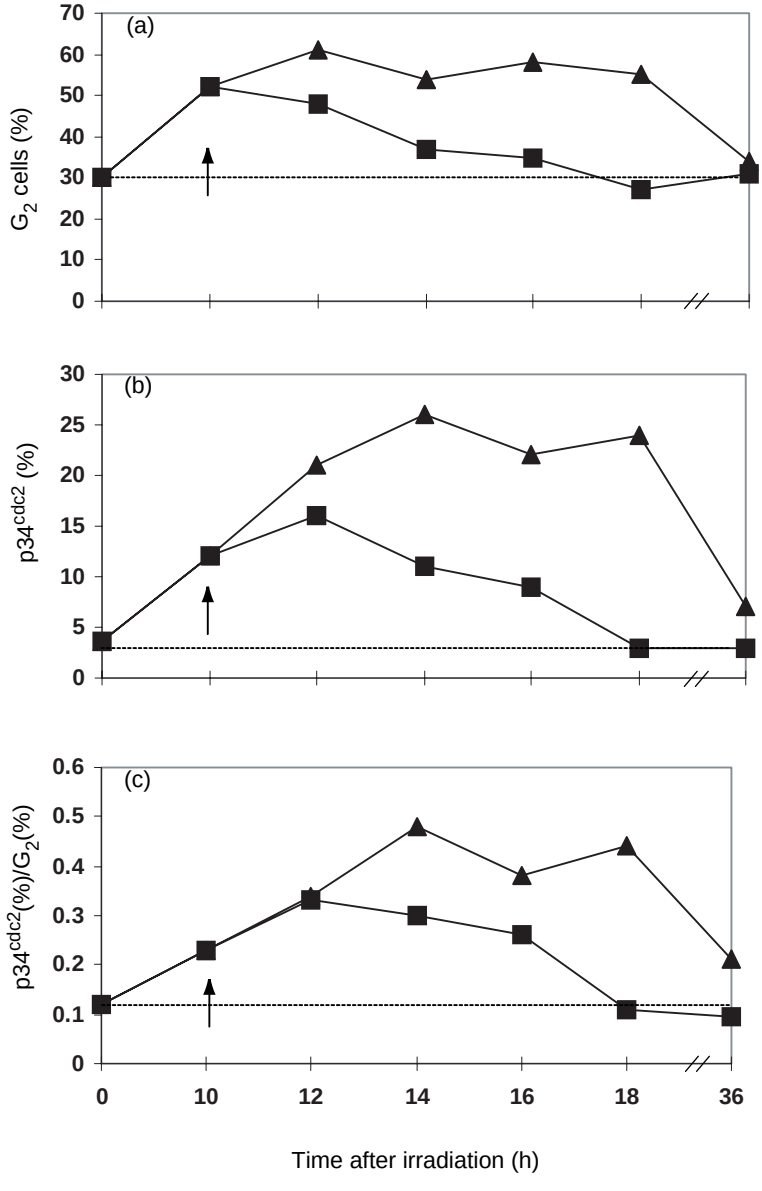

Figure 3. (a) Increase of the G_2 fraction during expression of the G_2 block and decrease as the block resolves gradually over 36 h (▲). Decline of the G_2 fraction over 6–8 h in response to addition of 2 mM pentoxifylline (■). (b) Increase of $p34^{cdc2}$ expression during expression of G_2 block (▲). Rapid decline of p34 after addition of 2 mM pentoxifylline within 8 h (■). (c) $P34^{cdc2}/G_2$ ratios plotted over 36 h after irradiation. Control cells (irradiation only) showing that restoration normally requires over 36 h (▲). Addition of 2 mM pentoxifylline and decline of ratio (■). Dotted lines show control levels of G_2, $p34^{cdc2}$ and $G_2/p34^{cdc2}$ in (a), (b) and (c), respectively.

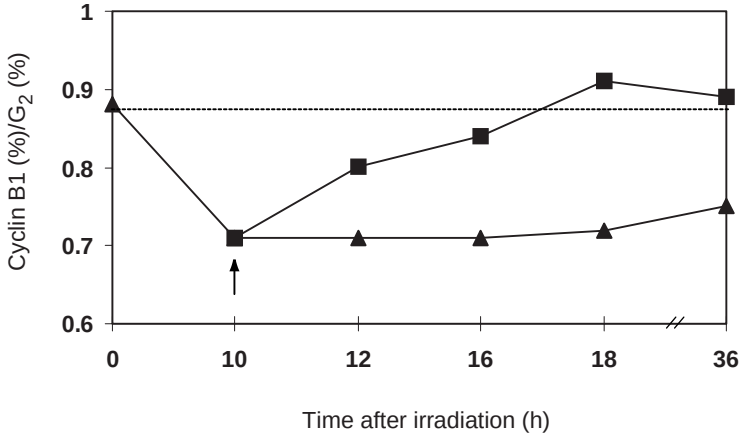

Figure 4. Cyclin B1/G_2 ratios plotted over 36 h after irradiation. Decline and recovery of ratio as the block resolves (▲). Addition of 2 mM pentoxifylline at 10 h post-irradiation results in rapid recovery of ratio within 6–8 h (■). Dotted line shows control ratio of cyclin B1/G_2 in an unperturbed cell cycle.

of pentoxifylline to whole cells at the time of maximum G_2 block results in a rapid restoration of the control cyclin B1/G_2 ratio within ~7 h which then remains at control levels for up to 35 h. In the absence of pentoxifylline, these ratios were not fully restored within 35 h (Figure 6a). In nuclei (Figure 6b), the G_2 block abrogation by pentoxifylline induces a sharp drop in cyclin B1 expression after 18 h. This suggests that cyclin B1 crosses the nuclear membrane after G_2 block abrogation and re-enters the cytoplasm where it is probably degraded by ubiquitin-mediated proteolysis (Pines 1999). Identical data trends were observed in A549 human lung carcinoma cells (not shown).

DISCUSSION

These results show that irradiation-induced DNA damage has a profound influence on the expression of cyclin B1 and $p34^{cdc2}$. These are constituents of the MPF complex and thus control the G_2/M transition. A prior study demonstrated that cyclin B1 expression declines after exposure of HeLa cells to ionizing irradiation and that the cyclin levels are restored to control levels as a result of the G_2 block abrogation by pentoxifylline (Theron & Böhm 1998). In the present study, $p34^{cdc2}$ and cyclin B1 expression were measured under identical conditions. The rise in $p34^{cdc2}$ expression (Figure 3b) is in agreement with results from Smeets, Mooren & Begg (1994) who reported an increase of the hyperphosphorylated inactive form of $p34^{cdc2}$ based on Western blots. Since G_2 block formation is associated with an increase in the number of G_2 cells and the level of $p34^{cdc2}$ (Figure 3a,b), it was decided to use the p34/G_2 ratios to assess changes of $p34^{cdc2}$ expression relative to the number of cells in G_2 (Figure 3c). G_2, $p34^{cdc2}$ and the ratio of $p34^{cdc2}/G_2$ show a maximum at 10–14 h. Addition of pentoxifylline rapidly restores the $p34^{cdc2}$/G2 ratio to the control values within 8 h (Figure 3a,b,c). It therefore can be safely concluded that $p34^{cdc2}$ is upregulated in response to DNA damage. It is possible that this upregulation results in the expression of a hyperphosphorylated, inactive form for the duration of the G_2 block (Smeets *et al.* 1994).

It appears that the existence of two MPF components enhances the sensitivity of the complex to small changes of the concentration of either constituent. This would result in an

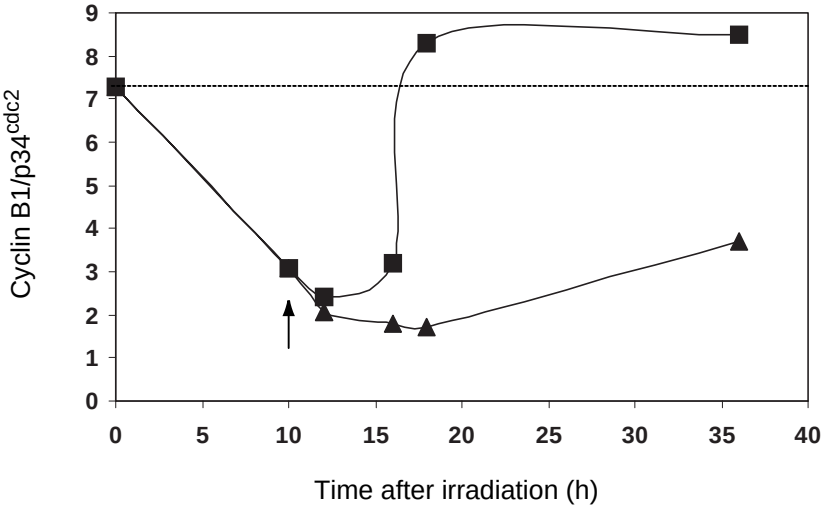

Figure 5. Cyclin B1/p34^{cdc2} ratios plotted over 36 h after irradiation. Decline and recovery of ratio after irradiation (▲). Abrogation of G_2 block by 2 mM pentoxifylline and rapid restoration of ratio (■). Dotted line shows control ratio of cyclin B1: p34^{cdc2} in an unperturbed cell cycle.

'all-or-nothing' reaction resembling a switch. The required changes in substrate concentrations do not follow the normal Michaelis–Menten enzyme kinetics but are characterised by an ultrasensitive response (Ferrel & Machleder 1998; Koshland 1998). In ultrasensitive kinetics, the maximum velocity is achieved by a 1–4-fold change in substrate concentration, rather than the 80-fold change observed in normal Michaelis–Menten kinetics (Koshland 1998). The switch-like response has previously been attributed to the translocation efficiency of the cyclin/cdk complex (Ferrel 1998). Our data suggest that changes in the concentration of cyclin B1 and p34^{cdc2} indeed act in concert and like an ultrasensitive switch (Figure 5). Translocation of the complex accross the nuclear membrane would further ensure rapid control of mitotic entry. The change of the cyclin B1/p34^{cdc2} ratios closely resembles other ultrasensitive reactions, e.g. the cooperativity of O_2 binding by haemoglobin (Koshland 1998). Our experiments only assess the expression of the required amounts of cyclin B1 and p34^{cdc2} and do not address the additional activation expected from MPF phosphorylation and dephosphorylation. We suggest that the restoration of the control ratio of cyclin B1/p34^{cdc2} expression by pentoxifylline forms a crucial part of the mechanism of action of this G_2 block abrogator.

When we assessed the influence of block abrogation on the subcellular location of cyclin B1 no significant differences were seen between cyclin B1 expression in whole cells and in nuclei, although overall levels seem consistently higher in whole cells. A sharp increase in cyclin B1 expression occurs in nuclei during G_2 block abrogation by pentoxifylline. Subsequently (after ~8 h), the B1 levels in nuclei falls sharply to even below the levels of irradiated control cells. This is most likely followed by ubiquitin-mediated proteolysis of the cyclin B1 required for exit from mitosis (King *et al.* 1996). It thus appears that pentoxifylline prompts mitotic entry and also exit from mitosis. The premature entry into mitosis could be the explanation for the enhanced cell kill observed when methylxanthines are combined with irradiation or chemotherapeutic drugs (O'Connor 1996; Husain *et al.* 1998; Li *et al.* 1998). In p53 mutant cells, which cannot invoke a G_1 block, a second challenge given at the G_2

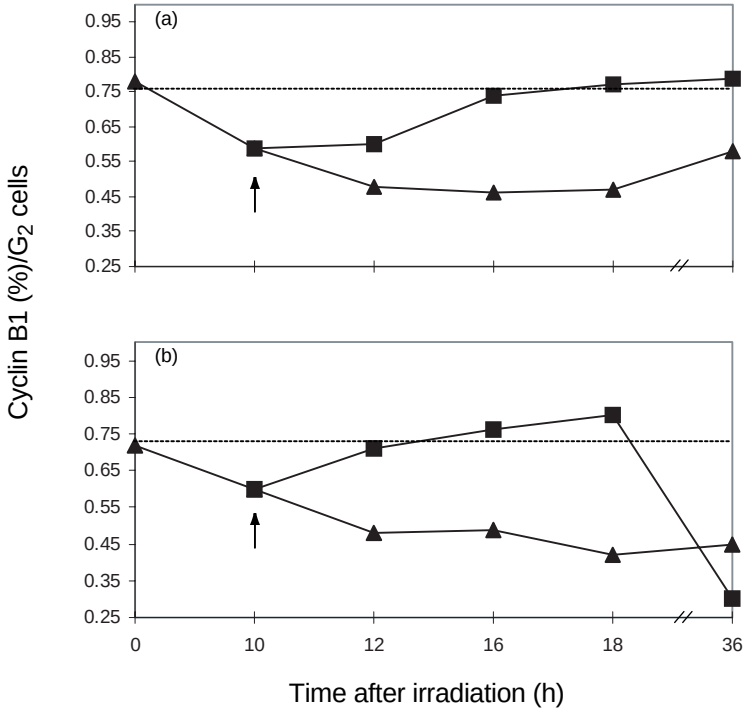

Figure 6. Cyclin B1/G_2 ratios plotted over 36 h post-irradiation for whole HeLa cells (a) and isolated HeLa nuclei (b). Recovery of G_2 block in whole cells (a) and nuclei (b) after irradiation (▲). Effect of 2 mM pentoxifylline in whole cells (a) and nuclei (b) on cyclin B1/G_2 ratio (■). The sharp drop in nuclear cyclin B1 between 18 and 36 h after irradiation suggests nuclear export. Dotted line shows ontrol ratio of cyclin B1: G_2 in an unperturbed cell cycle.

maximum results in even greater dose enhancement (Wang *et al*. 1996; Binder, Serafin & Böhm 1999).

These data suggest that G_2 block abrogation by pentoxifylline does not result in cell cycle perturbations and the uncoupling of mitosis, but in the resumption of normal cell cycle progression. These results are consistent with the hypothesis that the cell cycle is regulated in dimensions of both time (the timely synthesis and proteolysis of regulatory proteins) and space (localizing these regulators to the correct site) and that methylxanthines like pentoxifylline act at both levels to disrupt the G_2 cell cycle delay. The manipulation of cell cycle checkpoints remain an important topic for strategies in anti-cancer therapy.

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