Hypoxia-induced irreversible S-phase arrest involves down-regulation of cyclin A

J. Seim^{*1}, P. Graff^{*1}, Ø. Åmellem[†], K. S. Landsverk[‡], T. Stokke[‡] and

E. O. Pettersen^{*}

^{*}Department of Physics, the Biophysic group, University of Oslo, [†]Dynal Biotech ASA and [‡]Department of Biophysics, The Norwegian Radium Hospital, Oslo, Norway

Received 30 June 2003; revision accepted 25 September 2003

Abstract. We have studied hypoxia-induced cell cycle arrest in human cells where the retinoblastoma tumour suppressor protein (pRB) is either functional (T-47D cells) or abrogated by expression of the HPV18 E7 oncoprotein (NH1K 3025 cells). All cells in S phase are immediately arrested upon exposure to extreme hypoxia. During an 18-h extreme hypoxia regime, the cyclin A protein level is down-regulated in cells of both types when in S-phase, and, as we have previously shown, pRB re-binds in the nuclei of all T-47D cells (Åmellem *et al.* 1996). Hence, pRB is not necessary for the down-regulation of cyclin A during hypoxia. However, our findings indicate that re-oxygenation cannot release pRB from its nuclear binding following this prolonged exposure. The result is permanent S-phase arrest even after re-oxygenation, and this is correlated with a complete and permanent down-regulation of cyclin A in the pRB functional T-47D cells. In contrast, both cell cycle arrest and cyclin A down-regulation in S phase are reversed upon re-oxygenation in non-pRB-functional NH1K 3025 cells after prolonged exposure to extreme hypoxia. Our results indicate that pRB is involved in permanent S-phase arrest and down-regulation of cyclin A after extreme hypoxia.

INTRODUCTION

Different degrees of hypoxia arrest cells in different parts of the cell cycle. In this report we investigate the effects of extreme hypoxia (< 4 p.p.m. O₂) under which respiration is inhibited (Froese 1962). Under such conditions, cells in S phase immediately halt DNA synthesis, while cells in G₁ are either arrested at the pRB checkpoint or proceed to late G₁ before they become arrested at the oxygen-dependent checkpoint (subsequently referred to as the O₂ checkpoint). The molecular mechanism controlling this latter arrest point is unknown, but has been suggested to involve p27 (Gardner *et al.* 2001). This halt has also been shown to be independent of p53 and pRB and to operate in both normal and malignant cells (Graeber *et al.* 1994; Åmellem *et al.* 1996). Those arrested at the O₂ checkpoint enter S phase shortly after reoxygenation (1–1.5 h),

Correspondence: Pål Graff, Department of Physics, the Biophysic group, University of Oslo, PO Box 1048 Blindern, 0316 Oslo, Norway. Tel.: 47 22 85 56 52; Fax: 47 22 85 56 71; E-mail: pal.graff@fys.uio.no

¹These authors contributed equally to this work.

suggesting that molecules involved in initiation of S phase are either maintained under hypoxia or rapidly restored upon reoxygenation (Pettersen *et al.* 1986; Åmellem & Pettersen 1993; Åmellem *et al.* 1996). Hypoxia-induced cell cycle arrest in S phase has been shown to be a consequence of at least two levels of control. First, DNA replication is stopped due to specific inhibition of oxygen-dependent enzymes and inhibition of replicon initiation (Probst *et al.* 1999; Angus *et al.* 2002; Graff *et al.* 2002) and, secondly, the cell cycle checkpoint machinery is activated (in all cell cycle phases), leading to dephosphorylation of pRB and degradation of cyclins (Krtolica *et al.* 1998; Ludlow *et al.* 1993; Åmellem *et al.* 1996; Stokke *et al.* 1997; Sever-Chroneos *et al.* 2001).

Intra-S-phase checkpoints can be triggered under stress, and by DNA damage – conditions that promote dephosphorylation and nuclear binding of pRB in S phase. (Knudsen *et al.* 2000; Åmellem *et al.* 1996) The potential mechanisms by which pRB controls S phase progression are multifold. In the present study we focus on the ability of underphosphorylated pRB to inhibit S-phase progression by repressing transcription of genes required for DNA replication, such as cyclin A. Cyclin A plays a role in replicon initiation and is necessary for activation of the DNA polymerase δ -dependent DNA elongation machinery (Cannella *et al.* 1997; Bashir *et al.* 2000; Koundrioukoff *et al.* 2000; Sever-Chroneos *et al.* 2001). Cyclin A/cdk2 activity is also required for nucleosome assembly during DNA replication. (Keller & Krude 2000) Apart from direct involvement in DNA replication, cyclin A/cdk2-mediated phosphorylation of the E2F-1/DP-1 complex, disrupting its DNA binding activity, is required for S phase completion. (Krek *et al.* 1995)

In the present study, we measured the protein levels of cyclin E and cyclin A (i) in relation to cell-cycle stage under extreme hypoxic conditions and (ii) following re-oxygenation and correlated it to the nuclear binding of pRB. In pRB-functional cells we have previously shown that pRB is activated in cells in S-phase during a prolonged hypoxic exposure, and that they remain activated for several hours following re-oxygenation (Åmellem *et al.* 1996). We now demonstrate that cyclin A is degraded after this hypoxia, irrespective of pRB-functionality. Following re-oxygenation, cyclin A is re-produced in non-pRB-functional, but not in pRB-functional cells rendered hypoxic in S-phase.

MATERIALS AND METHODS

Cell cultures

Cells of the human breast cancer cell line T-47D (Keydar *et al.* 1979) were grown as monolayer cultures in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 10% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 200 units/l insulin and 1% penicillin/streptomycin (Gibco). The doubling time for T-47D cells has been established to be 37 h (Stokke *et al.* 1993). Cells of the human cervical carcinoma cell line NHIK 3025 (Nordbye & Oftebro 1969; Oftebro & Nordbye 1969) were grown as monolayer cultures in Minimum Essential Medium (MEM), supplemented with 15% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). The doubling time for NHIK 3025 cells has been established to be 18 h (Koritzinsky *et al.* 1998). NHIK 3025 cells bear the human papillomavirus 18 (HPV18) (Åmellem *et al.* 1998). Cell cultures were kept in exponential growth at 37 °C in air containing 5% CO₂ by re-culturing twice a week.

Hypoxic cell cultures

The technique of producing and maintaining various hypoxic conditions in cell cultures has been described previously (Pettersen & Lindmo 1981). Briefly, cells were seeded in 70-mm (Ø) glass

dishes (Anumbra, Prague, Czech Republic) 1 day before the experiment, and incubated in a CO₂ incubator. At the appropriate time, the glass dishes were taken from the CO₂ incubator into a walk-in incubator room at 37 °C. The medium content in each dish was reduced from 10 to 3.5 ml, and the dishes were placed, without lids, in a stainless steel chamber. Deoxygenation was achieved by continuous flushing of the chamber with a gas mixture (Hydro Gas, Oslo, Norway) of 97% N₂, 3% CO₂ and < 4 p.p.m. O₂ at 37 °C using the set-up described earlier (Løvhaug *et al.* 1977). Final level of O₂-concentration in the chamber was established roughly 12 min after the start of flushing. Untreated control populations were kept in the CO₂ incubator during the experiment.

SDS-PAGE and western immunoblotting

Cells were lysed with Laemmli sample buffer and proteins were separated on an 8% discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel with a 4% stacking gel. The proteins were transferred on to Hybond-P (Amersham Biosciences, Little Chalfont, UK) nitrocellulose membrane using Mini Trans Blot (Bio-Rad Laboratories, Hercules, CA, USA) tank blotting with blotting buffer containing 2.5 mM Tris (pH 8.3), 19.2 mM glycine and 20% methanol. The membranes were then blocked at 4 °C overnight in Tris-buffered saline (TBS) containing 5% non-fat dried milk and 0.1% Tween-20, before immunolabelling with 1 µg/ml monoclonal mouse antibodies against cyclin A or cyclin E (supplied by Pharmingen, San Diego, USA, BF683 and HE12, respectively). For each, the secondary antibody (peroxidase conjugated rabbit anti-mouse) was supplied by Dako (Glostrup, DK). Detection of bound antibodies was performed with ECL (Amersham Biosciences) according to manufacturers protocol.

Extraction, fixation and staining for measuring contents of DNA and cyclin A

Harvested cells were washed once with phosphate-buffered saline (PBS), fixed in 80% ethanol, and stored at -20 °C. Subsequently, all steps were performed at 0 °C. Cells were washed three times in PBS, and a three-layer procedure for staining cyclin A was employed. Cells were resuspended in 50 µl mouse monoclonal anti-cyclin A antibody (Pharmingen, BF683). As secondary antibody, biotinylated horse anti-mouse IgG₁ (HAM) (Vector Laboratories, Burlingame, CA, USA) was used and bound antibodies were detected with streptavidin-FITC (Amersham Biosciences). DNA staining was performed with 2 µg/ml Hoechst 33258.

Flow cytometry

Stained cells were measured in a FACStar^{PLUS} flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with one argon and one krypton laser (Spectra Physics, California, USA) tuned to 488 nm and UV, respectively. The following parameters were measured: forward light scatter (FSC), side scatter (SSC), FITC fluorescence intensity (cyclin A), integrated Hoechst 33258 intensity (DNA content), Hoechst 33258 fluorescence pulse height, and Hoechst 33258 fluorescence pulse width. Data were gated on FSC versus SSC and Hoechst 33258 fluorescence pulse area versus pulse width, to exclude debris and aggregates of cells, respectively (not shown in the figures). Green fluorescence intensities were calibrated with fluorescent beads prior to each experiment, such that the FITC fluorescence intensity measured in different experiments could be compared.

Radioactive pulse labelling of DNA

The cells were pulse labelled with 1.0 µCi [methyl-³H]thymidine ([³H]Tdr) for 10 min. After the pulse labelling with [³H]Tdr cells were incubated twice (15 min each) with normal media. Pulse labelling was performed either prior to the hypoxic treatment or 6 h after re-oxygenation. Cells

were harvested and stained for flow cytometry as described above. Flow cytometry was used to sort S-phase cells that were either cyclin A⁺ or cyclin A⁻. After sorting, cells were fixed in 2 ml 10% perchloric acid (PCA) for 15 min at 0 °C and washed three times with 2% PCA. Cells were then incubated with 2 ml 0.4% sodium deoxycholate (NaDOC) for 30 min at 37 °C before they were transferred to polyethylene vials and supplied with 8 ml of scintillation liquid (Emulsifier-safe, Packard Instrument Company, Meriden, CT, USA). Radioactivity was measured in a Tri-Carb 2100TR (Packard Instrument Company) scintillation counter.

RESULTS

Hypoxia down-regulated the expression of cyclin A irrespective of pRB status

Immunoblotting showed no change in the cyclin E protein level following 18 h hypoxic treatment of T-47D cells (Fig. 1). In contrast, immunoblotting of cyclin A protein (Fig. 1) was at a reduced level in T-47D cells following 18 h exposure to extreme hypoxia compared with the aerobic level. This level was partially restored 6 h after re-oxygenation. However, as cyclin A expression varies through the cell cycle, and as hypoxia is known to induce arrest in different phases, the observed changes could have been caused simply by a redistribution of cells in the cell cycle during hypoxia and after re-oxygenation.

To resolve this question, we measured cyclin A and DNA content simultaneously by flow cytometry (Figs 2 and 3). Rate of increase in expression of cyclin A during S phase was constant in both cell lines (Figs 2 and 3, panel a), increase of cyclin A starting at the onset of S phase. The amount of cyclin A protein reached its maximum in cells with a G₂/M DNA content. This subpopulation consisted of both cyclin A-positive and cyclin A-negative cells, representing cells in G₂ and mitosis, respectively (Figs 2 and 3, panel g). After 18 h of exposure to extremely hypoxic conditions, a decrease in cyclin A protein level was observed in mid to late S and G₂ phases of cells from both cell lines (Figs 2 and 3, panels c and g). It therefore appeared that cyclin A expression under aerobic conditions, as well as the reduction during hypoxia, were regulated in a similar manner in both cell lines, regardless of the functional status of pRB.

Figure 1. The effect of extreme hypoxia (< 4 p.p.m. O₂) on cyclin A and cyclin E expression in T-47D cells. Exponentially growing T-47D cells were exposed to aerobic conditions (A) or 18 h of extreme hypoxic conditions (H, R). Hypoxic cells were re-oxygenated and subsequently exposed to aerobic conditions for 0 h (H) or 6 h (R). Whole cell lysates were prepared, proteins were separated by SDS-PAGE (8%) and immunoblot analysis was performed as described in MATERIALS and METHODS.

Figure 2.

Figures 2 (above) and 3 (overleaf). Flow cytometric 2-parameter histograms (DNA versus cyclin A) of three different cell populations of either T-47D cells (Figure 2) or NHIK 3025 cells (Figure 3). The original 2-parametric histograms of each of the three populations are shown in the left column (i.e. panels a, c and h). Respectively, these represent: exponentially growing aerobic cells (a) serving as a control, cells treated for 18 h with < 4 p.p.m. O_2 (c) and cells given the same hypoxia-treatment, but in addition re-oxygenated for 6 h. All the other panels represent extracts of the 2-parametric histograms. The mid column (i.e. panels b, d, i and k) are DNA histograms where b is the total DNA-histogram of the aerobic cells (from panel a), (d) is the total DNA histogram of hypoxic cells (from panel c) and (i) and (k), respectively, are DNA-histograms of the subpopulations in (h) having low or high amount of cyclin A (denoted, respectively, A⁻ and A⁺). The right column (panels e, f, g, j and l) represent cyclin A histograms extracted from regions of panels (a), (c) and (h) defined by gatings (or windows). Panels (e), (f) and (g) compare the cyclin A histograms of hypoxic (grey line) and aerobic (solid black line) cells with either G₁ (gate 1), S (gate 2) or G₂/M (gate 3) DNA content, respectively.

Figure 3.

Re-oxygenation did not restore cyclin A levels in the pRB-functional T-47D S-phase cells after prolonged exposure to extreme hypoxia

A difference in regulation of cyclin A expression between T-47D and NHIK 3025 cells was observed during the first 6 h after re-oxygenation following 18 h hypoxic treatment (Figs 2 and 3, panel h). In NHIK 3025 cells, cyclin A protein level was restored in all S-phase cells, constituting an S-phase subpopulation with a slightly over-expressed cyclin A content (Fig. 3 panel h).

Figure 4. The effect of extreme hypoxia on cyclin A expression. Median values of cyclin A-associated fluorescence normalized to the value found in G₂ cells of the aerobic sample as a function of cellular DNA index, i.e. the DNA content relative to that of cells in G₁-phase. T-47D cells (a, c) and NHIK 3025 cells (b, d) were exposed to aerobic conditions (●) or 18 h of extreme hypoxic conditions (< 4 p.p.m. O₂) (a, b: ○). Hypoxic cells were re-oxygenated and subsequently exposed to aerobic conditions for 6 h (c, d: ■, □). For re-oxygenated T-47D cells (c), the cyclin A⁺ (■) and cyclin A⁻ (□) subpopulations are shown separately (Fig. 2, panel h), whereas re-oxygenated NHIK 3025 cells (d) constitute a single population (■) (Fig. 3, panel h). The analysis of the cyclin A level through the cell cycle was performed on flow-cytometric data by gating six sections through G₁, S and G₂/M phases, representing subpopulations differing in DNA content. The median value of cyclin A-associated fluorescence was estimated for each section, and non-specific fluorescence of the control sample (no primary antibody added), gated in exactly equivalent sections, was subtracted. The resulting fluorescence values of aerobic samples were normalized to the value found in G₂ cells of each sample. For aerobic samples (●) each experimental point represents the mean ± SE of nine different experiments and correspondingly for hypoxic samples (○) five to seven different experiments. Each experimental point of the cyclin A⁺ (■) and cyclin A⁻ (□) subpopulations of re-oxygenated T-47D cells, represents a mean ± SE of two and four experiments, respectively. These values were obtained by first gating as indicated by the windows in Figs 2 and 3 (h). The curve for re-oxygenated NHIK 3025 cells (■) represents a single experiment.

In T-47D cells, two clearly distinct S-phase subpopulations were identified (Fig. 2, panel h), one with cyclin A levels comparable with that in re-oxygenated NHIK 3025 cells (cyclin A⁺) and the other containing much less or no cyclin A protein (cyclin A⁻).

Further analysis of the cyclin A protein level at different cell cycle stages was performed by gating sections through G₁, S and G₂/M phases, representing subpopulations differing in DNA content (see legend of Fig. 4). The median values of cyclin A-associated fluorescence in individual samples were estimated for each section, and corresponding values of non-specific (background) fluorescence were subtracted (Fig. 4). The analysis confirmed observations from the individual samples considered above: the rate of increase in the cyclin A level during S phase was fairly constant in both cell lines under aerobic conditions, and the reduction during hypoxia was statistically significant in mid-to-late S and G₂ phases in both cell lines. In addition,

Table 1. Disintegrations per minute per cell for cells labelled either prior to the hypoxic treatment or 6 h after re-oxygenation. Measurements were performed on S-phase cells that were sorted into two compartments, A⁺ and A⁻, as shown in Fig. 5

	Fraction	DPM per cell	DPM _{A⁺} /DPM _{A⁻}
Pulse prior to hypoxia	A ⁺	0.008	0.16
	A ⁻	0.051	
Pulse at 6 h after reoxygenation	A ⁺	0.144	11.0
	A ⁻	0.013

Figure 5. Gates for sorting of cells used in pulse labelling. T-47D cells were treated with 4 p.p.m. O₂ for 18 h and then re-oxygenated and subsequently exposed to aerobic conditions for 6 h. The cells were pulse labelled with [³H]Tdr for 10 min either prior to the hypoxic treatment or 6 h after re-oxygenation. Cells were fixed in 80% ethanol and stained for cyclin A as described above. Two fractions of interest were then sorted by flow cytometry. One fraction contained S-phase cells without cyclin A, denoted A⁻. The other fraction contained S-phase cells with cyclin A, denoted A⁺. The two fractions were then used for scintillation counting as described in MATERIALS AND METHODS.

background correction revealed that the cyclin A⁻ subpopulation of re-oxygenated T-47D cells contained very little, if any, cyclin A protein. In re-oxygenated NHIK 3025 cells, no cyclin A⁻ subpopulation was observed.

Cyclin A content in a fraction of cells in G₁-phase increased during hypoxia

Exposure to 18 h extreme hypoxia increased the distribution range of cyclin A protein content in cells of both lines in G₁-phase (Figs 2 and 3, panels c and e). In all our experiments, the hypoxic treatment induced accumulation of cyclin A in G₁ cells to higher levels than were observed under aerobic conditions. In T-47D cells the accumulation of cyclin A appeared to be restricted to only a small fraction of cells in G₁, while in the majority of G₁ cells cyclin A level was unaffected by hypoxic treatment (Fig. 2, panel e). In NHIK 3025 cells the accumulation appeared to be more evenly distributed among cells with a G₁ DNA content (Fig. 3, panel e), however, this fraction with increased cyclin A in G₁ during hypoxia, did not greatly affect average content of cyclin A in G₁ in either of the cell lines (Fig. 4). NHIK 3025 cells in G₁-phase that accumulated cyclin A during hypoxia, entered S phase in a highly synchronous manner within a few hours after re-oxygenation, and progressed through S at a typical rate concomitant with further increase in cyclin A protein level. Consequently, the cyclin A protein levels in these re-oxygenated NHIK 3025 cells were much higher than those found in

untreated, aerobic cells (Figs 2 and 3, panel h). With T-47D cells, a subpopulation, here denoted cyclin A⁺ contained more than the aerobic level of cyclin A in S, while the other subpopulation, denoted cyclin A⁻ showed no increase in cyclin A and continued to contain little, if any, cyclin A.

Only cyclin A⁺ cells synthesized DNA after re-oxygenation and no cyclin A⁺ cells were derived from cyclin A⁻ S-phase cells.

Cells were pulse labelled with [³H]Tdr as described in MATERIALS and METHODS either prior to hypoxic treatment or 6 h after re-oxygenation. The cells were then sorted by flow cytometry. Two fractions, the S-phase cyclin A⁺ and cyclin A⁻, were collected as shown in Fig. 5, and incorporated [³H]Tdr was measured by scintillation counting. The measured radioactivity for the cells pulse labelled prior to hypoxic treatment was six times greater in the cyclin A⁻ fraction than in the cyclin A⁺ fraction, indicating that cells in the cyclin A⁻ fraction had been in S phase at the onset of hypoxia, while cells in the cyclin A⁺ fraction had not. Thus, the cyclin A⁺ fraction was recruited from cells that had been in either G₁ or G₂/M phase at the onset of hypoxia. Furthermore, cells labelled 6 h after re-oxygenation had 12 times more [³H]Tdr-activity in the cyclin A⁺ fraction than in the cyclin A⁻ fraction (Table 1). This indicated that only cells in the cyclin A⁺ fraction were able to replicate DNA.

DISCUSSION

In the present study, we correlated nuclear binding of pRB and the protein levels of cyclin E and cyclin A in relation to cell cycle stage under extreme hypoxic conditions and following re-oxygenation in T-47D and NHIK 3025 cells. We showed that cyclin A was degraded in both cell lines after prolonged hypoxic exposure, indicating that this process was independent of pRB. After re-oxygenation NHIK 3025 cells and T-47D cells in G₁-phase restored their cyclin A content and continued DNA synthesis. However, T-47D cells in S phase during hypoxia could not re-produce cyclin A and were irreversibly arrested in S phase, indicating that pRB was involved in permanent S-phase arrest and down-regulation of cyclin A in these conditions. As the two cell types differ in many respects, there is a possibility that variations other than pRB-competence could also play a role, but the data still strongly points to the direction of pRB being a prime operator in this regulation.

Regulation of pRB and cyclin A in S phase during hypoxia

Dephosphorylation of pRB in S phase during hypoxia has previously been correlated with down-regulation of cyclin A (Sever-Chroneos *et al.* 2001). In the present study, we observed an over-all reduction in cyclin A following 18 h of extreme hypoxia. Broken down into the various cell cycle phases, however, the picture was more complex. The reduction was solely limited to S and G₂ phases. In G₁ a small fraction of cells had more cyclin A than control G₁-cells although this did not affect the average content of cyclin A in G₁ following prolonged hypoxia. Down-regulation of cyclin A has been tightly linked with pRB-function. It has, for example, been demonstrated that introduction of phosphorylation site-mutated RB proteins (PSM-RB) into S-phase cells lacking functional pRB leads to transcriptional repression and down-regulation of cyclin A protein levels (Knudsen *et al.* 2000).

Unexpectedly, we here observed similar patterns of cyclin A expression in pRB-functional T-47D cells and non-pRB-functional NHIK 3025 cells immediately after prolonged exposure to extreme hypoxia (Figs 2 and 3). The cyclin A protein levels in mid-to-late S phase were reduced

in both cell types (Fig. 4), that is, pRB seemed not to be needed for the initial down-regulation of cyclin A. If pRB regulates cyclin A expression only at the level of transcription, the effect of extreme hypoxia therefore seemed to be mediated mainly through potentially pRB-independent post-transcriptional processes (translation, protein and mRNA degradation).

Diverging cyclin A regulation between the two cell types first became apparent after cells were re-oxygenated following the hypoxic treatment, resulting in no increase, but rather a clear decrease in cyclin A in pRB-functional T-47D cells arrested in S-phase by hypoxia, whereas non-pRB-functional NHIK 3025 cells in S phase restored their cyclin A protein level.

Hypoxia-induced cell cycle arrest in S phase in T-47D cells is irreversible

In a previous study we reported that T-47D cells were irreversibly arrested in S phase following a prolonged (18 h) exposure to extreme hypoxia, and that this correlated with irreversible nuclear binding of pRB (Åmellem *et al.* 1996). Here, we have shown that these irreversibly arrested S-phase cells permanently down-regulate cyclin A. The cells which progressed through S phase after re-oxygenation with a high cyclin A content were arrested in G₁ during hypoxia as shown by [³H]Tdr pulse labelling prior to hypoxic treatment (Table 1). However, with [³H]Tdr pulse labelling after hypoxic treatment, most of the radioactivity was found in the cyclin A⁺ subpopulation (Table 1), suggesting that only in these cells did DNA synthesis occur. That is, T-47D cells arrested in S phase during hypoxia remained arrested in S phase following re-oxygenation and did not restore their cyclin A level, whereas T-47D cells arrested in G₁-phase during hypoxia were able to re-initiate DNA synthesis.

Complete degradation of cyclin A occurred only in non-progressing T-47D cells within 6 h after re-oxygenation following prolonged hypoxic treatment. In contrast, non-pRB-functional NHIK 3025 cells in S phase restored cyclin A expression after re-oxygenation. We also observed that the subpopulation of cells with low levels of cyclin A remained equal in size to subpopulations that maintained nuclear binding of pRB and that remained arrested in S phase following re-oxygenation. These observations suggest that all three subpopulations were identical and that hypoxia-induced, permanent S-phase arrest mediated by pRB involved a permanent down-regulation of cyclin A. Taking into account the ability of pRB to repress cyclin A transcription, and the observation that cyclin A is permanently down-regulated only in the pRB-functional cell type (T-47D), we therefore suggest that the potential inhibitory action of nuclear-bound pRB on the cyclin A promoter may be responsible for the inability of re-oxygenated T-47D cells in S phase to reproduce cyclin A, once the protein is degraded.

It has been suggested that the hypoxia-induced, permanent S-phase arrest of T-47D cells is mediated by nuclear binding of pRB (Åmellem *et al.* 1996). The question remains why re-oxygenation is unable to reverse this nuclear binding of pRB in S-phase cells. This is readily accomplished in cells arrested at the pRB checkpoint in G₁, so why not in S? The answer might relate to differences between early G₁ and S phase, in factors that control pRB phosphorylation following re-oxygenation. Cells in both phases contain nuclear bound pRB and lack cyclin A, suggesting that the initial (re-) phosphorylation of pRB must rely on kinase activity associated with cyclins E and D. We observed no down-regulation of cyclin E in T-47D cells even after a prolonged hypoxic exposure, and though not investigated in this study, the cdk2 protein level has been reported to be relatively unaffected by hypoxic stress (Krtolica *et al.* 1998; Krtolica *et al.* 1999; Gardner *et al.* 2001). Taking into account our observations that the cdk inhibitors p21^{Cip1} (Åmellem *et al.* 1998) and p27^{Kip1} (Åmellem, unpublished data) are not induced in S phase by hypoxia, we can therefore assume a relatively normal cyclin E/cdk2 activity in the arrested S-phase cells. Whether cyclin D/cdk4(6) activity is restored or pRB-directed phosphatase activity returns to a normal level in S-phase cells after re-oxygenation, is currently unknown. Taken

together, cdk activity present in permanently arrested S-phase cells may therefore cause partial re-phosphorylation of pRB after re-oxygenation, but, unlike the situation in G₁ cells, this may not be sufficient to relieve transcriptional repression of cyclin A or to ultimately disrupt nuclear binding of pRB. To follow this hypothesis, factors that co-operate with pRB to reinforce its nuclear binding and repressor activity in arrested S-phase cells might be sought. A possible candidate is the aromatic hydrocarbon receptor (AHR), which has been shown to associate with pRB and participate in pRB-mediated repression of cyclin A when triggered by ligands, some of which are endogenous (Puga *et al.* 2000; Strobeck *et al.* 2000). It might be speculated that DNA damage acquired during hypoxia in S phase might induce a ligand that triggers AHR, causing a reinforced binding of the RB protein in the cell nucleus compared with its binding in its normal growth suppressive function in G₁. This could explain the irreversibility of hypoxia-induced pRB-mediated S-phase arrest, and is also consistent with the effects observed with the DNA damaging agent cisplatin, which induces irreversible cell cycle arrest in a pRB-dependent manner (Knudsen *et al.* 2000).

ACKNOWLEDGEMENTS

The skilful technical assistance of Charlotte Borka is gratefully acknowledged. The present study was supported by the Norwegian Cancer Society.

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