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# Bcl-2 can Promote p53-Dependent Senescence versus Apoptosis without Affecting the G1/S Transition

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With the aim to identify events involved in the determination of p53-dependent apoptosis versus growth arrest, we used rat embryo fibroblasts expressing a temperature-sensitive mutant (tsA58) of the SV40 large tumour antigen (LT). Heat-inactivation of LT leads to p53 activation and commitment to a senescent-like state (REtsA15 cell line) or apoptosis (REtsAF cell line). We report that senescence is associated with high levels of the anti-apoptotic Bcl-2 protein and a cell cycle arrest in G1 phase, whereas apoptosis is associated with low levels of Bcl-2 and a cell cycle arrest in G2 phase. Here we show that Bcl-2, which can inhibit apoptosis and proliferation, turns the apoptotic phenotype into a senescent-like phenotype in G2 phase. This result suggests that Bcl-2-dependent inhibition of apoptosis could be crucial for the commitment to replicative senescence, whereas its ability to inhibit G1 progression would not be required.

*Key Words:* bcl-2; senescence; apoptosis; cell cycle.

Normal cells in culture exhibit a limited mitotic life-span [1]. After a finite number of divisions, cells stop to proliferate and cannot be induced to enter S phase or to divide. This process is known as replicative or cellular senescence. The use of DNA tumour viruses has been particularly important to identify effectors of replicative senescence. For instance, it has been shown that inhibition of the Rb and p53 proteins by the large T antigen of the SV40 virus (LT) is crucial to bypass replicative senescence and to trigger the rodent cells to proliferate indefinitely [2,3]. When hypophosphorylated, the Rb protein blocks cell proliferation in G1 phase [4]. This process becomes irreversible in senescent cells which ordinary fail to phosphorylate Rb [5]. The p53 protein would also be critical for replicative senescence at least because of its participation to Rb activation. Indeed, p53 up-regulates transcription of the *p21<sup>waf-1/cip1/sdi1</sup>* gene which encodes a cdk inhibitor that inhibits Rb phosphorylation [6]. In addition to its anti-proliferative effect, p53 can induce an intrinsic cell death program, termed apoptosis. p53 essentially triggers apoptosis in stress conditions, notably via the expression of several target genes which promote cell death [7]. For instance, p53 induces expression of *bax* which facilitates the release of pro-apoptotic factors from the mitochondria [8]. Bax belongs to the Bcl-2 family, which contains positive and negative regulators of apoptosis. Contrary to Bax, the Bcl-2 protein prevents cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types [9]. It has been suggested that a balance between expression of these pro- and anti-apoptotic homologues could determine the choice between cell survival and apoptosis [10]. In this way, it was previously reported that Bcl-2 may be important for cell survival during senescence [11]. More recent studies have shown that Bcl-2 also inhibits cell cycle progression in G1 phase [12], but the mechanisms underlying this new function are poorly understood.

In order to identify the events involved in the determination of p53-dependent growth arrest versus apoptosis, we used rat embryo fibroblasts expressing a temperature-sensitive mutant (tsA58) of LT. This mutant cannot interact with p53 at restrictive temperature. Thus, the cells are immortalised at permissive temperature (33°C), whereas heat-inactivation of LT leads to a loss of the immortalised state at restrictive temperature (39.5°C) [13]. We have isolated several cell lines which harbour distinct phenotypes at 39.5°C. Some of them exhibit a senescent-like phenotype, whereas others exhibit an apoptotic phenotype [14]. Here, we have analysed two model cell lines undergoing pseudo-senescence (REtsA15 cells) or apoptosis (REtsAF cells) at 39.5°C. We report that senescence of REtsA15 cells is associated with a cell cycle arrest in G1 phase as well as high levels of the anti-apoptotic Bcl-2 protein. Conversely, we show that apoptosis of REtsAF cells is associated with low levels of Bcl-2 and a cell cycle arrest in G2 phase. Since the overexpression of *bcl-2* turns the apoptotic phenotype into a senescent-like phenotype in G2 phase, our results suggest that Bcl-2-dependent inhibition of apoptosis could be crucial for the determination of replicative senescence, whereas its ability to inhibit cell cycle progression in G1 phase would be dispensable.

## MATERIALS AND METHODS

*Cell lines and cell culture.* Isolation of the REtsAF cell line has been described previously [13]. REtsA15 cell line was isolated at low cell density from a rat embryo fibroblast culture transfected with the tsA58 allele of the LT gene. REtsAF-Bcl-2 cell line is derived from REtsAF cells and overexpresses the human *bcl-2* gene [15]. Cell culture was performed as previously described [15].

*RT-PCR assay.* RNA were isolated using the guanidium isothiocyanate method [16]. To estimate the level of *bcl-2* and *bax* mRNA, RT-PCR was performed as previously described [17] using specific primers (*bcl-2*: forward primer 5'agccctgtgccacctgtggt 3', reverse primer 5'acgctttgccacgggtggtg3'; *bax*: forward primer 5'ggagcagctcggaggcg3', reverse primer 5'ttcttggtggatgcatcctg3'). Amplified products, were collected in the exponential phase (*bcl-2*: 143 bp / 25-30 cycles and *bax*: 158 bp / 32-35 cycles), separated on 10% acrylamide gels, transferred to hybond N+ membranes (Amersham) and hybridised with <sup>32</sup>P labelled specific probes. Quantification for each PCR was done using ImageQuant software after scanning the autoradiograms.

*Western-blot analysis.* SDS-page and Western blot were performed as previously described [15]. The following antibodies were used in this study: anti-Bax (N-19, Santa Cruz), anti-Bcl-2 (83-8B, MBL), anti-Rb (G3-245, Pharmingen) and anti-Waf-1 (C-19, Santa Cruz).

*Flow cytometry.* Flow cytometry measurements were performed with a XL3C flow cytometer (Beckman-Coulter France). For cell viability, cells were incubated with 0.2 µg/ml fluorescein diacetate (FDA) 30 min and 5 µg/ml propidium iodide (PI) 5 min prior analysis. FDA (Polysciences) is a nonfluorescent compound which becomes fluorescent (free fluorescein) when cleaved by esterases in living cells. PI (sigma) specifically penetrates in necrotic cells, after loss of their plasma membrane integrity. For cell cycle analysis, cells were fixed with ethanol 70% at -20°C, rinsed with PBS and incubated with PI 100 µg/ml and RNase (Roche) 0.25 mg/ml in PBS 15 min. Cell debris (including Sub-G1 cells) were excluded from cell cycle analysis. For measurement of BrdU incorporation, cells were incubated for 30 min with 30 µM BrdU (Sigma), fixed with ethanol 70% at -20°C and rinsed with PBS. Next, cells were incubated in HCl 2N 20 min at 37°C, rinsed with PBS, incubated with 0.5% tween 20, 0.5% NGS and 4% (v/v) of a rat anti-BrdU antibody (Sera-Lab) 1h, rinsed with PBS and incubated 30 min with 0.5% tween 20, 0.5% NGS (Dako) and 4% (v/v) of a FITC-conjugated anti-rat secondary antibody (Jackson). Finally, cells were rinsed in PBS and the percentage of BrdU (FITC) positive cells was determined.

## RESULTS

### *A Loss of the Immortalised State Leads to Distinct Phenotypes in REtsA15 and REtsAF Cells*

At permissive temperature (33°C), REtsA15 and REtsAF fibroblasts proliferate indefinitely. It was previously shown that heat inactivation of LT induces replicative senescence when cells have bypassed their normal proliferative potential [18]. Figure 1a shows that REtsA15 cells progressively stop to proliferate at restrictive temperature (39.5°C, left panel) and adopt an enlarged morphology (right panel), which is characteristic of cellular senescence *in vitro*. On the contrary, the number of REtsAF cells progressively decreases at 39.5°C and a photograph clearly shows that they are condensed and detached from their culture dish. A condensation and a loss of cell adhesion are morphological features of the programmed cell death, termed apoptosis. It was previously shown that the REtsAF cells are positive for TUNEL, a technique that reveals DNA fragmentation of apoptotic or necrotic cells [14]. In order to further characterise the phenotype of REtsAF cells at 39.5°C, we have used the FDA/PI staining method (see materials and methods) which permits to separate living cells from apoptotic or necrotic cells [19]. Living cells are FDA positive whereas FDA negative-dead cells can be divided into two populations: PI positive-necrotic cells and PI negative-apoptotic cells. Figure 1b shows that after 48h at 39.5°C, most of REtsAF cells are apoptotic whereas REtsA15 cells remain essentially alive. These results show that a loss of immortalisation can lead to a senescent-like state or to an apoptotic cell death, depending on the cell line.

### *bcl-2 is Differentially Expressed in REtsA15 and REtsAF Cells*

When activated in pathological conditions, p53 can regulate positively or negatively the expression of several genes from the *bcl-2* family. For instance, p53 stimulates the expression of the pro-apoptotic *bax* gene and conversely inhibits the expression of the anti-apoptotic *bcl-2* gene [20]. In order to determine if the ratio between Bax and Bcl-2 is correlated to REtsAF and REtsA15 cell viability, we have studied their expression in both cell lines. The results presented in Figure 2 show that the basal expression of *bax* at 33°C is the same in REtsAF and REtsA15 cells. At 39.5°C, when p53 is activated, there is a stimulation of *bax* transcription. Even though this stimulation appears to be faster in REtsAF cells, the Bax protein levels increase similarly in both cell lines. On the contrary, Figure 2 shows that the expression of *bcl-2* is strongly induced in REtsA15 cells at 39.5°C, whereas it is not in REtsAF cells and that both mRNA and protein levels of *bcl-2* are higher in REtsA15 cells at 33°C and 39.5°C. Therefore, our results suggest that higher amounts of Bcl-2 could inhibit REtsA15 apoptosis and promote replicative senescence at 39.5°C.

### *REtsA15 and REtsAF Cells Harbour Distinct Regulations of Cell Cycle at 39.5°C*

p53 has also the ability to up-regulate transcription of the *p21<sup>waf-1/cip1/sdi1</sup>* gene which encodes a cdki that inhibits G1/S transition [6]. A Western-blot analysis shows that the p21<sup>Waf-1/Sdi-1/Cip1</sup> protein strongly accumulates in both REtsAF and REtsA15 cell lines at

39.5°C (Fig. 3). Even though the p21<sup>Waf-1/Sdi-1/Cip1</sup> protein accumulation appears to be more rapid and intense in REtsA15 cells, this result clearly shows that p53 is actually activated in both cell lines at 39.5°C. In order to examine the influence of p21<sup>Waf-1/Sdi-1/Cip1</sup> accumulation on Rb phosphorylation, we have also performed a Western-blot analysis of Rb. Figure 3 shows that three forms of Rb which correspond to the hypophosphorylated (p105), phosphorylated (p110) and hyperphosphorylated (p115) Rb, can be distinguished at 33°C. Unexpectedly, the active p105 form of Rb seems to disappear rapidly in REtsAF cells at 39.5°C whereas it accumulates in REtsA15 cells, like p110/115. These results suggest that Rb activity at 39.5°C is different in REtsAF and REtsA15 cell lines and that a strong accumulation of p21<sup>Waf-1/Sdi-1/Cip1</sup> at 39.5°C is not sufficient to maintain hypophosphorylated Rb in REtsAF cells.

Next, we have performed a flow cytometric analysis at 33°C and 39.5°C to examine the effect of Rb phosphorylation on REtsA15 / REtsAF cell cycle regulation and DNA synthesis. We have first estimated the proportion of cells in S phase by measuring the incorporation of bromodeoxyuridine (BrdU). Figure 4a shows that the percentage of BrdU positive cells decreases at 39.5°C in both cell lines, suggesting that the REtsAF and REtsA15 cells both stop to proliferate. However, the inhibition of DNA synthesis is much more rapid in REtsA15 cells. Next, we have examined the distribution of REtsAF and REtsA15 cells in the different phases of cell cycle (Fig. 4b). This distribution is identical at 33°C. However, when shifted at 39.5°C, the REtsA15 cells essentially accumulate in G1 phase whereas REtsAF cells accumulate in G2/M phases (actually in G2 phase since chromatin is not condensed (data not shown)). These results are in agreement with the status of Rb in these cells, suggesting that Rb is not inhibited by LT at 39.5°C, like p53. They also raise the idea that cell survival could depend on cell cycle position at 39.5°C since a G1 block is associated with cell survival whereas a G2 block is associated with cell death. Finally, it is possible that the G2 arrest results from the expression of the *14-3-3-σ* and *GADD45* genes, which are other targets of p53 [21,22].

#### *Overexpression of bcl-2 in REtsAF Cells Turns the Apoptotic Phenotype into a Senescent-like Phenotype in G2 Phase*

The results described above raise the possibility that the amount of Bcl-2 as well as cell cycle regulation could influence the choice between replicative senescence and apoptosis upon p53 activation, in REtsA15 / REtsAF cells. In addition to its anti-apoptotic role, Bcl-2 has also been described to inhibit cell cycle progression in G1 phase [12]. Thus, it was of particular interest to determine if Bcl-2 was able to influence the phenotype observed at 39.5°C, in a cell cycle-dependent or independent manner. In order to test these hypotheses, we have used the REtsAF-Bcl-2 cell line which overexpresses the human *bcl-2* gene [15]. An FDA/PI analysis of REtsAF-Bcl-2 cells (Fig. 5a) clearly shows that Bcl-2 protects REtsAF cells from apoptosis at 39.5°C. Indeed, after 48h passed at restrictive temperature, there is 10% of cell death in REtsAF cells overexpressing *bcl-2* versus 90% in REtsAF cells (Fig. 1b). Interestingly, a photograph of REtsAF-Bcl-2 cells shows that surviving cells adopt a senescent-like morphology (Fig. 5b). Thus, *bcl-2* expression would not only inhibits apoptosis but would also allow cells to undergo senescence. Next, Figure 5c shows that the

accumulation of Bax and p21<sup>Waf-1/Sdi-1/Cip1</sup> is similar in REtsAF-Bcl-2 and REtsAF cells at 39.5°C. It also shows that hypophosphorylated Rb (p105) rapidly disappears at 39.5°C, like in REtsAF cells. Finally, Figure 5d and 5e show that *bcl-2* overexpression does not influence cell cycle regulation of REtsAF cells, since REtsAF-Bcl-2 cells still exhibit a delayed inhibition of DNA synthesis (versus REtsA15 cells) and accumulate in G2 phase. Thus, it appears that Bcl-2 could promote the determination of replicative senescence independently of the regulation of G1/S transition in this system. It also suggests that a senescent-like phenotype can be established in G2 phase and that the Bcl-2-dependent inhibition of apoptosis can be essential for the determination of replicative senescence.

## DISCUSSION

In this work, we point out that a loss of the immortalised state, involving p53 activation, can be associated with either a senescent-like state (REtsA15 cells) or apoptosis (REtsAF cells). The p53-induced senescent state, which is established within a few days, is probably different from classical replicative senescence obtained with primary cultured cells. For instance, we were unable to detect a SA- $\beta$ -gal (senescence associated  $\beta$ -galactosidase) activity [23]. However, we and others have shown that this process exhibits important molecular and cellular features of replicative senescence, such as p21<sup>Waf-1/Sdi-1/Cip1</sup> accumulation, maintenance of hypophosphorylated Rb, irreversibility of growth arrest in G1 phase and morphological changes [24].

In this article, we report that the Bcl-2 protein level is important in choosing between p53-induced senescence versus apoptosis at restrictive temperature. Indeed, our results clearly show that Bcl-2 inhibits apoptosis of REtsAF cells at 39.5°C and suggest that this inhibition allows the occurrence of replicative senescence, despite a growth arrest in G2 phase. The p53 protein can induce apoptosis via the induction of several target genes involved in the mitochondrial and the death receptor-induced apoptotic pathways. Several mediators of the p53-induced apoptotic response are pro-apoptotic members of the Bcl-2 family, such as Bax, Noxa and Puma which trigger cytochrome c release and activation of apoptosome [7]. Interestingly, we have previously shown that a shift of REtsAF cells at 39.5°C is associated with a decrease in mitochondrial membrane potential ( $\Delta\Psi_m$ ) [25], and that this decrease is inhibited by Bcl-2 overexpression [26]. Thus it seems that Bcl-2 inhibits the p53-dependent mitochondrial apoptotic pathway. The p53 protein can also promote apoptosis via a repression of survival genes, such as *bcl-2* and *IGF-1R* [27, 28]. Surprisingly, we found that p53 activation is correlated with an accumulation of Bcl-2 in REtsA15 cells. However, we have observed that in these cells, *bcl-2* mRNA level decreases in the first hours passed at 39.5°C and then increases during several days (data not shown). Thus, p53 may have a dual effect on Bcl-2 level: a short time inhibitory effect and a long time stimulatory effect.

We also report that there is a correlation between cell cycle regulation and cell fate at 39.5°C: senescence is correlated with a rapid inhibition of DNA synthesis and a growth arrest in G1 phase (REtsA15), whereas apoptosis is correlated with a delayed inhibition of DNA synthesis and a block in G2 phase (REtsAF). The fact that REtsAF-Bcl-2 cells seem to senesce in G2 phase suggests that the inhibition of G1/S transition would be dispensable for

the commitment to replicative senescence. It also indicates that Bcl-2 cannot promote a G1 block in these cells. This result could be related to the fact that Bcl-2-dependent inhibition of G1 progression was demonstrated with quiescent cells re-entering cell cycle [12], whereas REtsAF cells are cycling cells at 33°C. Hypophosphorylated Rb triggers a growth arrest in G1 phase via the inhibition of E2F transcription factors, that are required for S phase entry and DNA synthesis [29]. Interestingly, it has been reported that the E2F-1 protein also reinforces the pro-apoptotic activity of p53 [30]. Thus, contrary to REtsA15, an inability of the REtsAF cells to stop to proliferate in G1 phase, which is correlated to an Rb hyperphosphorylation, could favour their own death through the activation of E2F-1. Therefore, it would be of particular interest to test the involvement of cell cycle regulation in apoptosis by determining if a G1 block imposed on REtsAF cells at 39.5°C (via a drug treatment or expression of an unphosphorylatable Rb mutant) could result in a senescent phenotype. The fact that Rb is rapidly phosphorylated in REtsAF cells, despite high amounts of p21<sup>Waf-1/Sdi-1/Cip1</sup>, remains unexplained. However, it seems likely that this process could originate in a cyclin/cdk overactivation or a p21<sup>Waf-1/Sdi-1/Cip1</sup> mutation.

Finally, it is noteworthy that *bcl-2* can promote replicative senescence whereas it was first identified as an oncogene involved in a follicular lymphoma. This paradox could be related to the fact that both commitment to replicative senescence and oncogenesis would necessitate inhibition of apoptosis.

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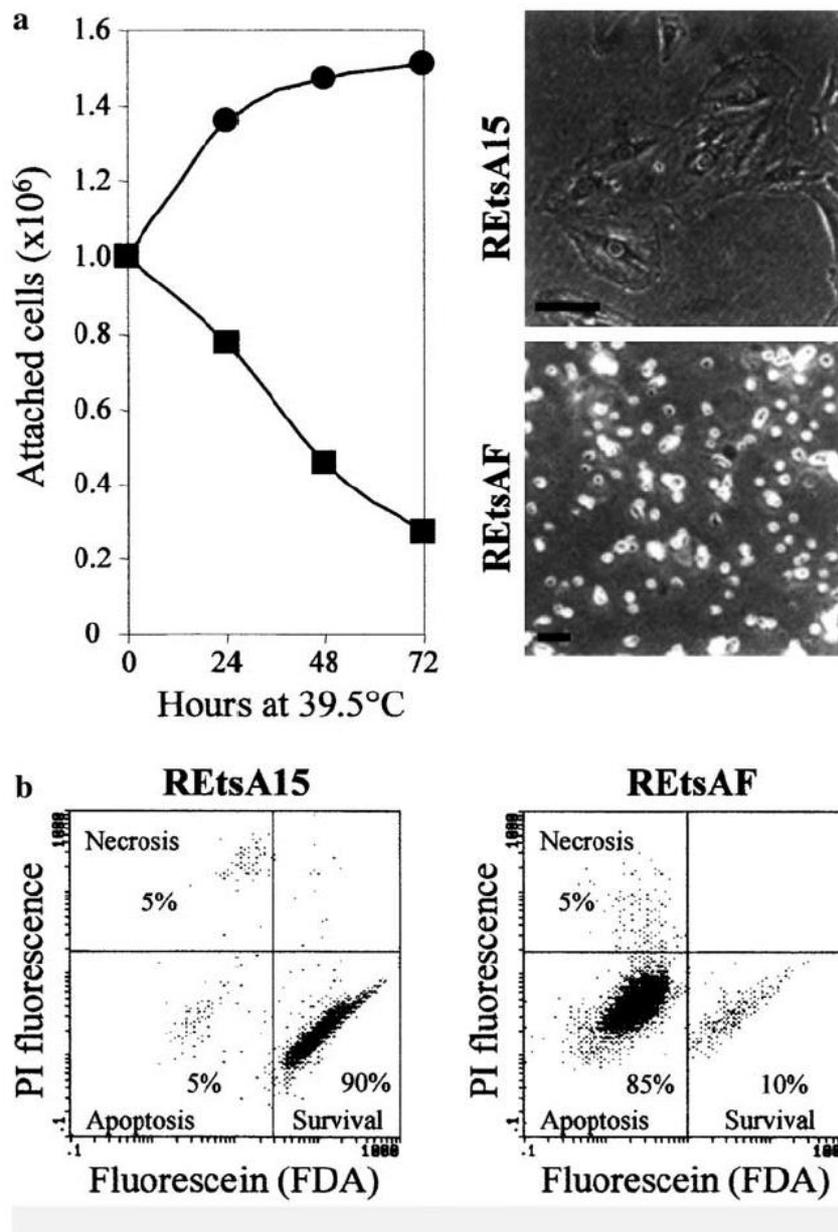
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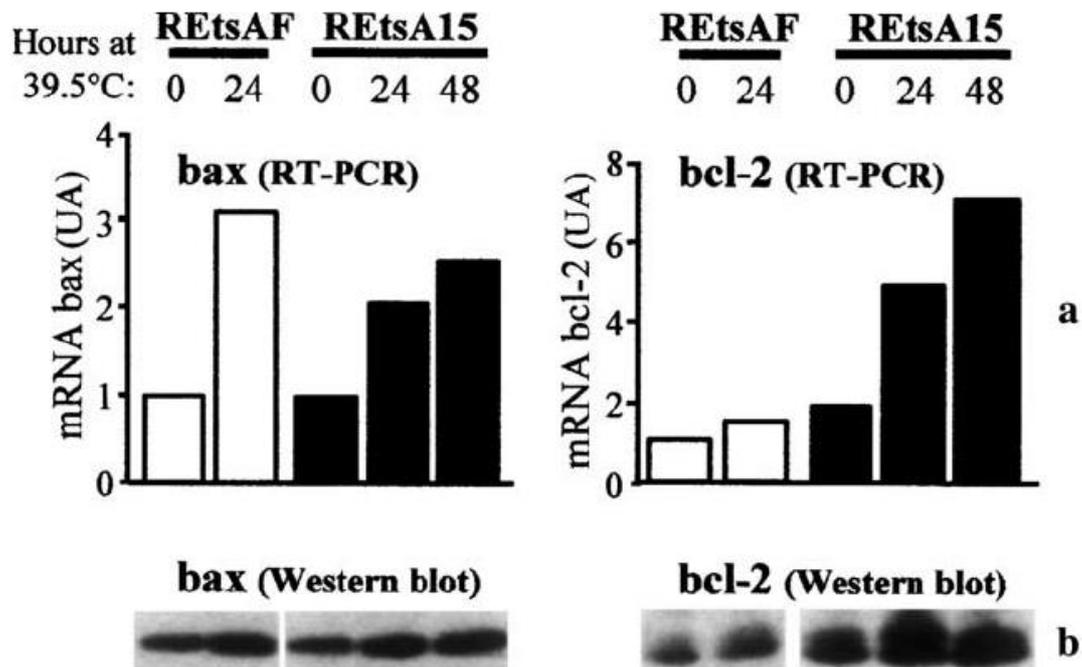
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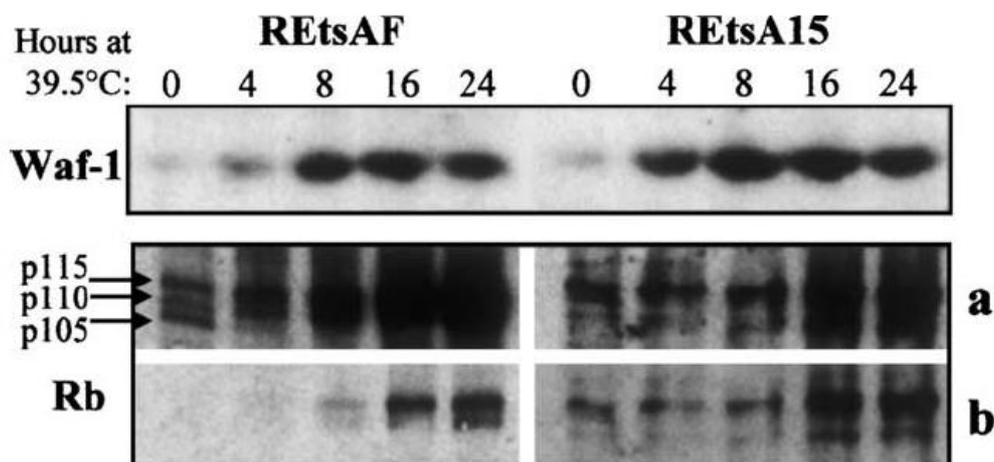
FIGURES



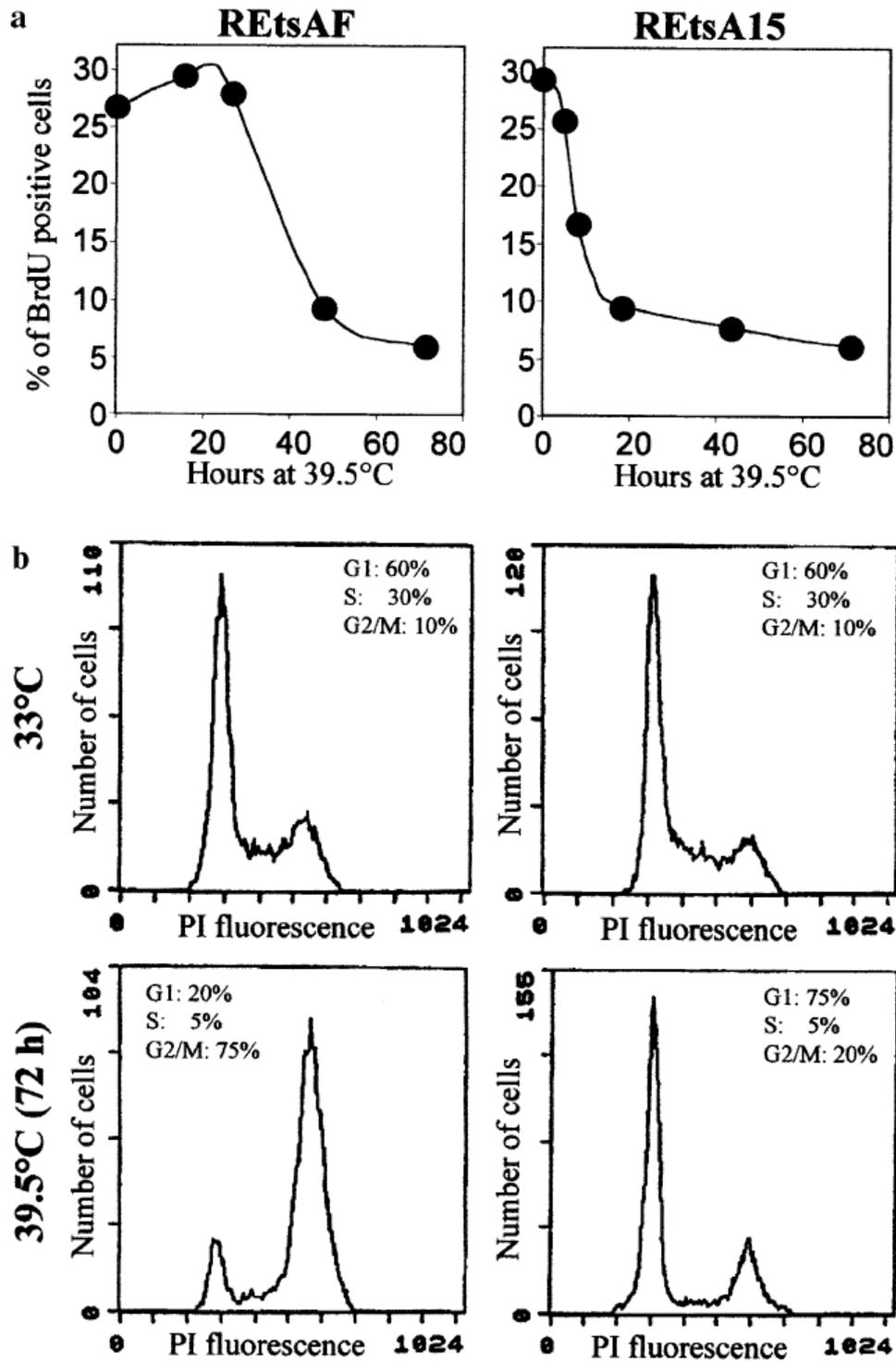
**FIG. 1.** Analysis of REtsA15 and REtsAF phenotype at 39.5°C. (a) REtsA15 and REtsAF cells were incubated at 39.5°C. REtsA15 cells (●) progressively stop to proliferate and harbour a senescent-like morphology whereas REtsAF cells (■) are detached from the culture dish and harbour an apoptotic-like phenotype (photographs were taken 48h after shift at 39.5°C). (b) A flow cytometric analysis of cell death was performed on both cell lines after 48h at 39.5°C. The cytograms obtained with REtsA15 and REtsAF cells show three distinct populations of cells: surviving, apoptotic and necrotic cells. REtsAF cells undergo a massive apoptotic cell death while most of REtsA15 cells remain alive.



**FIG. 2.** Analysis of *bax* and *bcl-2* expression in REtsAF and REtsA15 cells. RT-PCR and Western-blot analysis were performed to study expression of the *bax* and *bcl-2* genes. RT-PCR was used to characterise mRNA levels (a). For *bax*, similar basal levels were found in both cell lines whereas higher levels were observed for REtsAF cells at 39.5°C. For *bcl-2*, higher levels were observed for REtsA15 at 33°C and 39.5°C. Western-blot analysis (b) reveal that the Bax protein equally accumulates in both cell lines at 39.5°C whereas the amount of Bcl-2 is always superior in REtsA15 cells.



**FIG. 3.** Analysis of  $p21^{Waf-1/Sdi-1/Cip1}$  expression and Rb phosphorylation. Western-blot analysis were performed to characterise the level of  $p21^{Waf-1/Sdi-1/Cip1}$  protein and Rb phosphorylation at 33°C and 39.5°C. The  $p21^{Waf-1/Sdi-1/Cip1}$  protein accumulates in both cell lines at 39.5°C. The analysis of Rb also shows that it accumulates in both cell lines at 39.5°C. A long time exposure (a) allows to distinguish the hypophosphorylated (p105), phosphorylated (p110) and hyperphosphorylated (p115) forms of Rb in both cell lines at 33°C. A short time exposure (b) shows that p105, the hypophosphorylated and active form of Rb disappears in REtsAF cells at 39.5°C whereas it accumulates in REtsA15 cells.



**FIG. 4.** Analysis of DNA synthesis and cell cycle regulation in REtsAF and REtsA15 cells. DNA synthesis was estimated by measuring BrdU incorporation at 33°C and 39.5°C by flow cytometry. The percentage of BrdU positive cells strongly decreases in both REtsA15 and REtsAF cells at 39.5°C but decreases more rapidly in REtsA15 cells than in REtsAF (a). The distribution of REtsAF and REtsA15 cells in the different phases of cell cycle, is identical at 33°C (b). At 39.5°C, the proportion of REtsAF and REtsA15 cells in S phase decreases but REtsAF cells accumulate in G2/M phases whereas REtsA15 cells essentially accumulate in G1 phase.

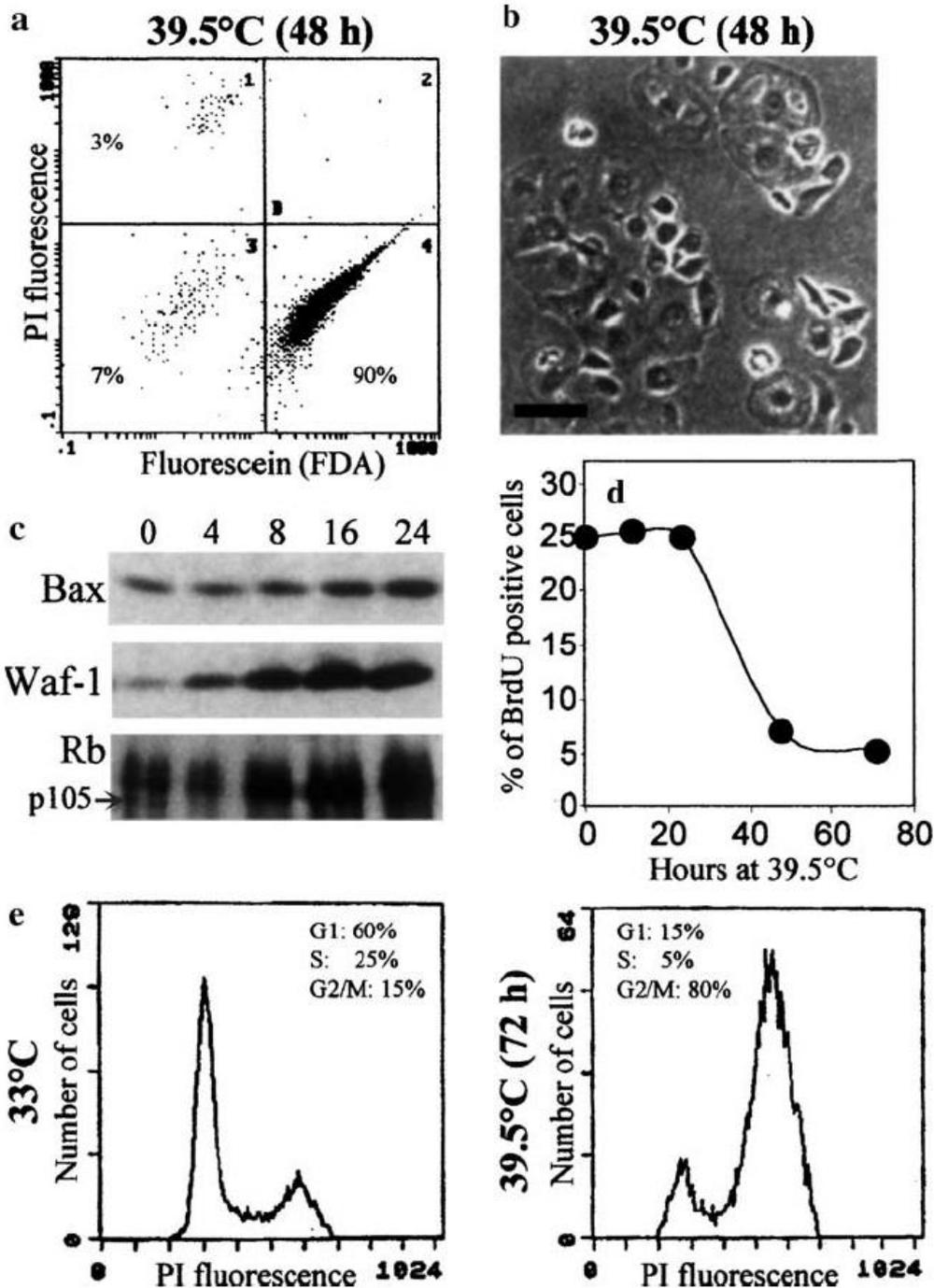


FIG.

5. Characterisation of the REtsAF-Bcl-2 phenotype at 39.5°C. Analysis of cell death (a) reveals that REtsAF-Bcl-2 cells at 39.5°C are essentially surviving cells. A photograph of REtsAF-Bcl-2 cells at 39.5°C (b) shows a senescent-like morphology and a Western-blot analysis shows a small accumulation of Bax, a strong accumulation of p21<sup>Waf-1/Sdi-1/Cip1</sup> and an hyperphosphorylation of Rb, similar to those observed in REtsAF cells at 39.5°C. Finally, *bcl-2* overexpression does not seem to modify significantly DNA synthesis (d) and cell cycle regulation (e) when compared to Figure 4.