Bcl-2 activates a programme of premature senescence in human carcinoma cells

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The apoptosis regulator Bcl-2 has been shown to modulate cell-cycle progression, favouring a quiescent state over a proliferative state, in both normal and tumour cells. We show here that constitutive expression of Bcl-2 in human carcinoma cells results in a cell-cycle arrest that within a few days can become irreversible. Arrested cells acquire a senescent-like phenotype, which consists of several characteristic morphological alterations and increased activity of senescence-associated β-galactosidase. The induction of the premature senescence programme is mediated by inhibition of Cdk2 kinase activity, and $p27^{KIP1}$ is required to maintain the senescent phenotype. We propose that the ability to activate an endogenous premature senescence programme allows Bcl-2 to suppress tumour growth. These results suggest that the down-regulation of Bcl-2 expression, which has been observed during the development and progression of human carcinoma, is related to the ability of Bcl-2 to severely hamper the growth of carcinoma cells and to induce a permanent cell-cycle arrest, with the features of senescence.

Key words: Bcl-2, carcinoma, Cdk2, $p27^{KIP1}$, p130, senescence.

INTRODUCTION

Tissue homoeostasis in eukaryotic organisms depends on a delicate equilibrium between cell growth and cell death. Many proteins that critically regulate cell division, in fact, can also induce programmed cell death [1–4] and, vice versa, several components of the apoptotic machinery may influence the cell cycle. For instance, apoptosis regulatory proteins of the Bcl-2 family have been shown to modulate cell-cycle progression. In particular, overexpression of the anti-apoptotic members of this family in B and T cells delays entry into S-phase [5–7] and accelerates withdrawal from the cell cycle [8,9]. In contrast, overexpression of pro-apoptotic members such as Bax or Bad in T cells of transgenic mice increases the number of cycling thymocytes, favouring entry into S-phase [6,10]. The ability of Bcl-2 to interfere with cell-cycle progression is not limited to lymphoid cells, but has also been observed in murine fibroblasts [7,11]. Overexpression of the *bcl-2* gene in normal, quiescent cells retards entry into the cell cycle, but does not affect growth of cells that are continuously cycling [7,8]. Therefore Bcl-2 can regulate the transition between the quiescent and the cycling state in normal cells. However, evidence exists to suggest that Bcl-2 can have a pronounced anti-mitotic effect on continuously cycling tumour cells [7,12]. This anti-proliferative property has been used to explain the putative tumour-suppressor activity of Bcl-2, which has been observed in transgenic mouse models of the mammary gland [13,14] and hepatic carcinogenesis [15,16]. In addition, elevated Bcl-2 levels have been associated with both a reduced proliferation rate and favourable prognosis in several human carcinomas, and a selective down-regulation of Bcl-2 has been observed during tumour progression in various human cancers [17–20].

Mammalian cells carry an intrinsic molecular programme that limits their multiplication. Replicative senescence has been described in normal cells as a permanent, irreversible cell-cycle arrest, which ensues at the end of their genetically defined lifespan [21]. Senescent cells remain viable indefinitely and acquire both a distinctive morphology and the expression of specific phenotypic markers, such as the acidic, senescence-associated β-galactosidase (SA-β-gal). Cell lines derived from tumours are generally capable of extended proliferation: indeed, limitless replicative potential represents one of the *de novo* acquired capabilities of tumour cells, and the ability to repress senescence pathways appears to contribute to tumorigenesis [22]. Accordingly, restoration of senescence regulatory pathways in tumour cells rapidly elicits senescence [23–25]. These observations suggest that, in several immortal tumour-derived cell lines, the genetic programme of senescence is repressed, but not lost, in such a way that it can be reactivated by expression of critical regulators.

Although tumour-suppressor activity of Bcl-2 protein has been observed in several cellular systems, the molecular mechanisms by which Bcl-2 affects the growth of carcinoma cells remains poorly defined. In the present study we analyse the growth-inhibitory effects elicited by Bcl-2 in human carcinoma cells. We first describe the ability of Bcl-2 to induce a senescent-like phenotype in human endometrial carcinoma cells. We then analyse the effects of inducible Bcl-2 expression in a human lung carcinoma cell line, H1299. We demonstrate that Bcl-2 can induce a permanent cell-cycle arrest, with features of cellular senescence. The induction of the premature senescence programme is mediated by a $p27^{KIP1}$-dependent inhibition of Cdk2. We also show that elevated $p27^{KIP1}$ levels are required to maintain the senescent phenotype. We propose that the ability to induce senescence contributes to the action of Bcl-2 in suppressing tumour growth.

EXPERIMENTAL

Cell cultures and drug treatment

HEC1B human endometrial carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD, U.S.A.).

Abbreviations used: HA, haemagglutinin; dn-K2, dominant-negative, HA-tagged form of Cdk2; DTT, dithiothreitol; FCS, fetal-calf serum; MEM, minimal essential medium; oligo, oligonucleotide; pRb, retinoblastoma protein; SA-β-gal, senescence-associated β-galactosidase.

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Cells were cultured in minimal essential medium (MEM; Sigma) supplemented with 10 % (v/v) FCS (fetal-calf serum), 2 mM glutamine, 0.5 unit/ml penicillin and 0.5 μg/ml streptomycin.

H1299 human lung carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured in RPMI 1640 medium, supplemented with 10 % (v/v) FCS, 2 mM glutamine, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM Hepes, 0.5 unit/ml penicillin and 0.5 μg/ml streptomycin. All media and cell culture reagents were purchased from Life Technologies.

Stably transfected HEC1B cells were cultured in MEM/10 % (v/v) FCS, containing 400 μg/ml G418 (Life Technologies). Stably transfected inducible H1299 cells were cultured in RPMI 1640 medium/10 % FCS containing 500 μg/ml G418 (Life Technologies) and 500 μg/ml hygromycin (Calbiochem).

For drug treatments, cisplatin and aphidicolin (Calbiochem) were dissolved in DMSO (5 mg/ml stock solution), whereas doxorubicin (Calbiochem) was dissolved in sterile water (5 mg/ml stock solution). Cells were synchronized by incubation with 5 μg/ml aphidicolin for 48 h.

The tetracycline analogue doxycycline (Clontech) was dissolved in sterile water (2 mg/ml stock solution) and used at a final concentration of 2 μg/ml.

Growth assay and colony-forming efficiency

Cells were plated in triplicate at 1×10^4 cells/well, in a 24-well plate in RPMI supplemented with 10 % FCS. After 16 h, cells were treated with or without doxycycline (2 μg/ml). The cell number was assessed using a haemocytometer.

For colony-forming assays, cells were plated in triplicate at a density of 5×10^3 cells in 6 cm dishes. Colonies were stained with 1 % Methylene Blue in 50 % (v/v) ethanol.

Cell transfection

HEC1B cells and H1299 cells were stably transfected using Lipofectin reagent (Life Technologies), following the manufacturer's protocol. Routinely, 2 μg of DNA per 60 mm dish was used. Selection of transfected cells was performed by adding the antibiotic G418 (400 or 500 μg/ml) or hygromycin (500 μg/ml) or puromycin (1 μg/ml) to the medium 72 h after the addition of constructs. After 2 or 3 weeks, clones were picked and expanded for further analysis. Clones expressing the highest levels of transfected proteins were chosen for further analysis.

SA-β-gal activity

Staining for SA-β-gal was performed as described previously [26].

Immunofluorescence microscopy

Cells were grown on to gelatin-treated glass coverslips in 60 mm dishes, and were allowed to adhere for 48 h. Cells were fixed with ice-cold methanol and permeabilized with 0.2 % Tween 20 in TBS. Cells were blocked with 10 % FBS in TBS-T buffer [Tris-buffered saline (TBS) containing 0.1 % (v/v) Tween 20] for 15 min. Bcl-2 was detected by incubating the cells with anti-Bcl-2 monoclonal antibody at a dilution of 1:500 for 1 h. Cells were washed with TBS-T and then incubated with a 1:500 dilution of fluorescein-tagged goat anti-mouse secondary antibody (Santa Cruz Biotechnology). After washes with TBS-T, the coverslips were mounted on to a microscope slide using a 90 % solution of glycerol in TBS and analysed with a Zeiss Axioplan2 microscope.

DNA manipulation and plasmids

Unless differently specified, general DNA manipulation was performed according to standard procedures [27]. The pSFFV-Bcl-2 expression vector was kindly provided by Dr J. Ashwell (National Institutes of Health, Bethesda, MD, U.S.A.). The pcDNA1-HA-p130 expression vector (where HA represents haemagglutinin) was kindly provided by Gabriel Gil-Gomez [Institut Municipal d'Investigacio Medica (IMIM), Barcelona, Spain]. The dominant-negative Cdk2 vector was kindly provided by Dr S. van den Heuvel [28]. Full-length cDNA of the dominant-negative, HA-tagged form of Cdk2 (dn-K2) was subcloned into the *Pvu*II site in the $pTRE_2$-pur tetracycline-inducible expression plasmid. The integrity of all constructs was confirmed by DNA sequencing.

The Tet-On gene expression system was purchased from Clontech (Palo Alto, CA, U.S.A.). The system includes three vectors: pTet-On, $pTRE_2$ and pTK-Hyg. The pTet-On vector encodes a tetracycline-controlled transactivator (rtTA protein). The $pTRE_2$ vector contains the multiple cloning site to accept the target gene. The promoter region upstream from the multiple cloning site is silent in the absence of binding of rtTA to the *tet* operator. When the rtTA protein binds to the *tet* operator, expression of the target gene is switched on. Doxycycline allows rtTA binding to the promoter.

H1299 cells at $\approx$60 % confluence were transfected with pTet-On plasmid (2 μg of DNA per 60 mm dish). Transfected cells were selected in RPMI 1640 medium containing 10 % FBS and 500 μg/ml G418. G418-resistant clones were screened with the luciferase assay (see below). rtTA clones with high levels of luciferase induction (> 40-fold) were selected for the second transfection.

The full-length cDNA of human Bcl-2 was subcloned into the *Pvu*II site in the $pTRE_2$ tetracycline-inducible expression plasmid. The pTK-Hyg vector, which has a hygromycin-resistant gene, was used to select the stable transfectants. pTK-Hyg and $pTRE_2$-Bcl-2 plasmids were co-transfected in a 20:1 ratio; the Bcl-2-transfected cells were selected in RPMI medium containing 10 % FBS and 500 μg/ml G418+500 μg/ml hygromycin.

Transient transfection and luciferase assay

rtTA-expressing clones were seeded in 12-well dishes in RPMI medium containing 10 % FCS. After 16 h incubation, pTRE-Luc plasmid (0.5 μg), which contains an inducible reporter luciferase gene, was transfected into the cells. After 6 h, cells were then treated with or without doxycycline (2 μg/ml). After 48 h, luciferase activity was measured using a commercial luciferase assay system, according to the manufacturer's instructions (Sigma).

Western blot analysis

Total cell-protein preparations were obtained by lysing cells with Lysis buffer (1 mM EDTA/0.2 % Triton X-100/1 μg/ml aprotinin/170 μg/ml PMSF). Protein concentration was routinely measured with the Bio-Rad protein assay.

Nuclear extracts were prepared by lysing cells with 10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT (dithiothreitol), 1 μg/ml aprotinin and 170 μg/ml PMSF. Nuclei were washed extensively with the same buffer, and collected by centrifugation [400 ***g*** (2000 rev./min), 10 min]. Finally, nuclei were lysed with Lysis buffer.

Polyacrylamide gels [6–15 % (w/v)] were prepared essentially as described by Laemmli [29]. Molecular-mass standards were

from Pharmacia. Proteins separated on the polyacrylamide gels were blotted on to nitrocellulose filters (Hybond-C pure; Amersham Biosciences).

Filters were washed and stained with specific primary antibodies, and then with secondary antisera, conjugated with horseradish peroxidase (diluted 1:2000; Amersham Biosciences). Filters were developed using the ECL® Western blotting detection reagent (Amersham Biosciences) and were quantitatively estimated by scanning with ImageMaster VDS-CL.

The antibodies against Bcl-2 (100), Bcl-X (S-18), $p27^{Kip1}$ (C-19), $p21^{Cip1}$ (C-19), $p57^{Kip2}$ (E-17), retinoblastoma protein (pRb; C-15), E2F1 (KH95), E2F4 (C-20), cyclin E (M20), cyclin A (C-19), cyclin D_1 (C-20), cyclin D_3 (C-16), Cdk4 (C-22), Cdk6 (C-21), Cdk2 (M2) and Sp1 (PEP-2) were purchased from Santa Cruz Biotechnology; anti-Bax-α (13666E) antibody was from Pharmingen; anti-α-tubulin antibody was from Serotec; anti-$p15^{INK4b}$ (05-430) was from Upstate Biotechnology; antibodies specific for the phosphorylated state of Ser^{807} and Ser^{795} in pRb were from New England Biolab; and anti-$p27^{Kip1}$-HPRO (K25025) was from Transduction Laboratories.

Immunoprecipitations and kinase assays

For Cdk2 immunoprecipitation, total cell-protein preparations were obtained by lysing cells with ice-cold RIPA buffer [1× PBS/1 % Nonidet P40/0.5 % (w/v) sodium deoxycholate/ 0.1 % SDS] containing 1 μg/ml aprotinin and 170 μg/ml PMSF. Cells were disrupted by repeated aspiration through a 21-gauge needle. Protein concentration was routinely measured using the Bio-Rad protein assay. For co-immunoprecipitations, 125 μg of total cellular protein was incubated with 5 μg of anti-Cdk2 agarose-conjugate antibody, followed by incubation at 4 °C overnight with mixing. Immunoprecipitates were collected by centrifugation at 500 ***g*** (2500 rev./min) for 5 min at 4 °C, which were then washed 4 times with RIPA buffer. The immunoprecipitates were fractionated on SDS/PAGE and immunoblotted with anti-$p27^{Kip1}$-HPRO antibody. Blots were re-probed with anti-Cdk2 antibodies as a loading control.

For the kinase assay, 125 μg of total cellular protein was incubated with 10 μg of anti-Cdk2 antibody (M2; Santa Cruz Biotechnology) for 1 h at 4 °C. Immunocomplexes were precipitated overnight at 4 °C with Protein G plus agarose (Santa Cruz Biotechnology). Kinase activity was routinely assayed by incubation with 5 μg of histone H1, 100 μM ATP and 5 μCi of [γ-^{32}P]ATP in kinase buffer [100 mM Tris/HCl (pH 7.4)/20 mM $MgCl_2$/2 mM DTT]. Samples from the kinase assay were resolved on an SDS/15 %-polyacrylamide gel, stained with Coomassie Blue to visualize the histone H1 bands, dried and exposed for autoradiography. Where required for quantification, the same samples were analysed by Western blotting and then probed with anti-Cdk2 antibodies as a loading control.

For E2F immunoprecipitation, nuclei were isolated as described previously, and were subsequently lysed with IP buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 mM DTT and 25 % glycerol containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 200 μM sodium orthovanadate, 1 mM pyrophosphate and 10 mM NaF). Protein concentration was routinely measured by the Bio-Rad protein assay. Nuclear proteins (200 μg) were incubated with 10 μg of anti-E2F4 antibodies, and were then incubated at 4 °C overnight with mixing. Immunocomplexes were collected with Gamma Bind G-Sepharose (Amersham Pharmacia Biotechnology) by centrifugation at 5900 ***g*** (8000 rev./min) for 15 s, and washed 4 times with IP buffer. The immunoprecipitates were fractionated on SDS/PAGE and immunoblotted with either anti-p130 or anti-pRb antibodies. Blots were re-probed with anti-E2F4 or anti-E2F1 antibodies.

Flow cytometry analysis

Samples for flow-cytometric analyses were prepared essentially as described by Nicoletti et al. [30]. Samples were analysed for DNA content on a Beckman Coulter FACS XL.

Apoptosis assay

Flow-cytometric analyses of apoptotic cells were performed as described by Nicoletti et al. [30]. Cells were pre-treated with 2 μg/ml doxycycline for 24 h, and then exposed to either 5 μg/ml cisplatin or 0.5 μg/ml doxorubicin. After 72 h, cells with sub-G_1 DNA content were quantified by flow cytometry. Uninduced cells were used as a control.

Antisense oligo (oligonucleotide) treatment

Phosphorothioate antisense oligos and matched randomized control oligos were synthesized by Biognostik (Göttingen, Germany). The sequence of the $p27^{KIP1}$ antisense oligo (TGG CTC TCC TGC GCC) corresponded to nt 305–320 of the human $p27^{KIP1}$-coding sequence (GenBank® accession no. AF247551).

H1299 cells at ≈60 % confluence were transfected using LIPOFECTAMINE™ 2000 Reagent (Life Technologies), following the manufacturer's protocol. A concentration of 2 μM oligos was used in all experiments.

RESULTS

Effects of Bcl-2 overexpression in human endometrial carcinoma cells

In order to assess the role of Bcl-2 on proliferation in human carcinoma cells, we initially transfected a human endometrial adenocarcinoma cell line, HEC1B, with a Bcl-2 expression vector and selected for stable transfectants by growing cells in the presence of G418 for 15 days. Control cells were transfected with pSFFV-neo empty vector. Expression of the transgene was assessed by Western blotting (results not shown) and by immunofluorescence (Figure 1A). There was no detectable immunofluorescence signal above background in control cells.

Overexpression of Bcl-2 in HEC1B cells resulted in distinctive morphological alterations. While parental and control cells that were transfected with empty vector grew in polygonal, epithelial-like fashion (Figure 1B), Bcl-2-overexpressing cells assumed a large and flat morphology, with abundant cytoplasmic vacuolization (Figure 1C). In addition, Bcl-2 transfectants showed a marked reduction in saturation density, as compared to controls. These phenotypic changes have been frequently observed in senescent cells. Therefore we analysed the activity of SA-β-gal in both control and Bcl-2-overexpressing cells. SA-β-gal is a specific marker of cellular senescence, that allows the distinction between senescent, quiescent and terminally differentiated cells to be made [26]. The Bcl-2 transfectants did express SA-β-gal (Figure 1D). Approx. 30 % of Bcl-2-overexpressing cells were positive for SA-β-gal staining, whereas the number of positive cells was very low in both parental HEC1B cells and vector-transfected control cells (Figure 1E). These observations suggest that constitutive overexpression of Bcl-2 results in a senescent-like phenotype in HEC1B cells.

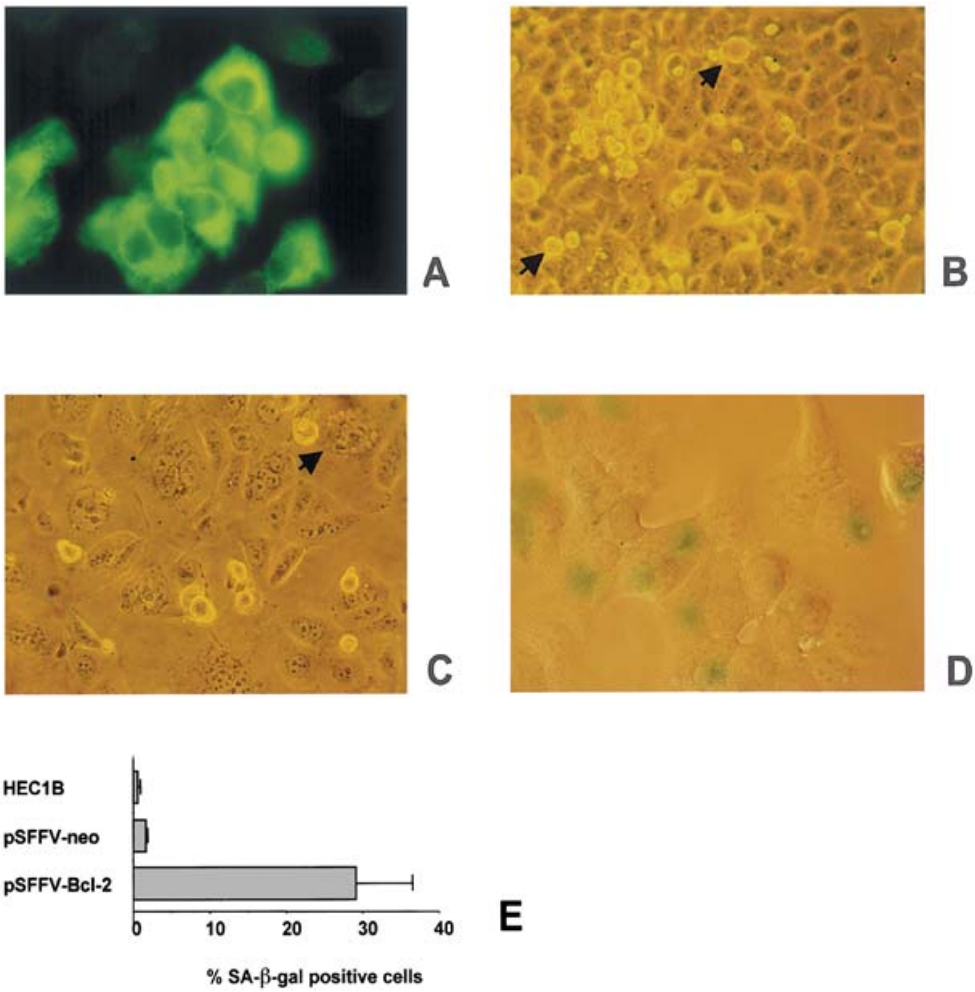

Figure 1 Morphological alterations and SA-β-gal staining in HEC1B cells overexpressing Bcl-2

HEC1B cells were photographed using a Nikon TMS microscope at 200× magnification. (**A**) HEC1B cells that stably overexpress Bcl-2 were immunostained with an anti-Bcl-2 monoclonal antibody followed by secondary fluorescein-conjugate antibodies. (**B**) Control parental HEC1B cells. The arrows indicate extra-cellular lumina. (**C**) Morphological alterations in Bcl-2-overexpressing cells. The arrow indicates cytoplasmic vacuolization. (**D**) Cells stained to detect SA-β-gal activity. (**E**) Triplicate samples of parental, neonatal or Bcl-2 HEC1B cells were plated at 2×10^5 cells per 60 mm dish and stained to detect SA-β-gal activity. The percentage of cells positive for SA-β-gal was determined by counting three random fields under a bright-field microscope. For each independent determination, a minimum of 200 cells were counted.

Generation of carcinoma cells inducibly expressing Bcl-2

We investigated the Bcl-2-dependent senescence programme in other carcinoma cells. Since the p53 gene is not required for Bcl-2-mediated growth inhibition [7,11], we selected a p53-null lung carcinoma-cell line, H1299. According to its growth-inhibitory activity, stable transfection of Bcl-2 resulted in a reduced cloning efficiency in H1299 cells, as well as in other tumour cell lines ([7,12] and results not shown). Hence, in order to avoid selecting for cells scarcely sensitive to the Bcl-2-dependent growth-inhibitory stimuli, we generated a tetracycline-inducible system of Bcl-2 expression in H1299 cells. Many clones inducibly expressing Bcl-2 were generated, and three clones (indicated hereafter as clones 4B1, 4B3 and 4B6) were selected for more extensive biochemical analysis. Figure 2(A) shows the time course of induction of Bcl-2 protein after the addition of the tetracycline analogue doxycycline. Induction of Bcl-2 occurred within 6 h, and maximum expression was obtained between 24 and 48 h. The effect of the inducer was specific, since neither Bcl-X_L nor Bax protein levels were affected significantly by treatment with doxycycline (Figure 2A).

In order to assess the functionality of the induced protein, we first examined the effect of Bcl-2 overexpression on both cisplatin- and doxorubicin-induced apoptosis. All clones demonstrated significantly increased resistance towards apoptotic stimuli after treatment with doxycycline, as determined by flow cytometry (results not shown). Hence the induced levels of Bcl-2 were sufficient to inhibit programmed cell death in H1299 cells.

Growth arrest and cell-cycle inhibition

In order to investigate whether Bcl-2 overexpression affects the proliferative ability of H1299 cells, growth was measured by cell counting. Whereas the clones grew exponentially in the absence of doxycycline, the growth rate was significantly decreased following induction of Bcl-2 (Figure 2B). We also examined the effect of Bcl-2 induction on the cell cycle. Asynchronously growing, untreated control cells and doxycycline-stimulated cells were analysed by flow cytometry. Bcl-2-induced clones rapidly accumulated in the G_1-phase of the cell cycle; a corresponding loss of cells from both S- and G_2/M-phases was also detected, but no

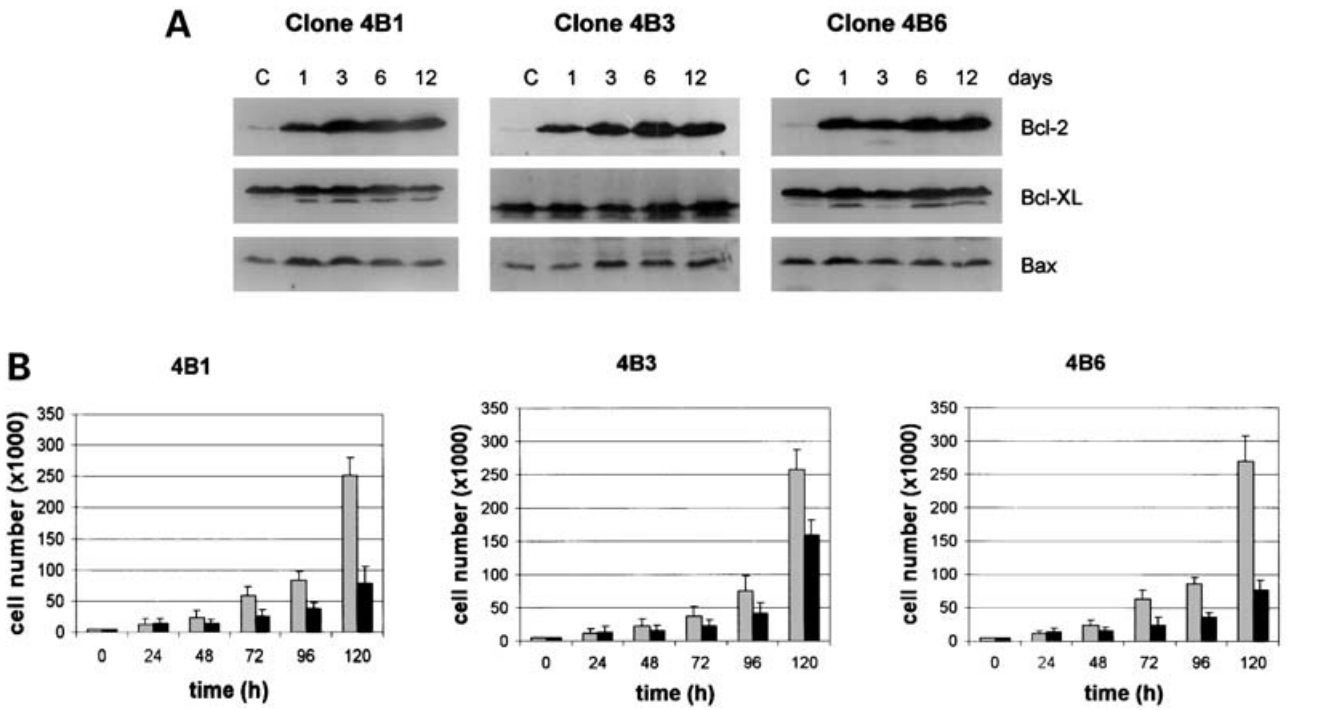

Figure 2 Inducible expression of Bcl-2 in H1299-derived clones (A), and cell proliferation in Bcl-2-overexpressing cells (B)

(**A**) Cells were treated with 2 μg/ml doxycycline for 1 to 12 days, and protein expression was detected by Western blotting. Extracts from uninduced cells were used as controls (labelled as 'C' in the Figure). The same filters were stripped and re-probed with anti-Bcl-X_L and anti-Bax antibodies. (**B**) 4B1, 4B3 and 4B6 cells were plated at 1×10^4 cells/well in 24-well dishes. After 16 h, cells were treated with doxycycline (2 μg/ml). Cell number was assessed on a haemocytometer. Data represent the average of three independent observations. Grey bars represent control cells; the black bars show doxycycline-treated cells.

Table 1 Effect of Bcl-2 induction on cell cycle

Clones 4B1, 4B3 and 4B6 were treated with 2 μg/ml doxycycline for 1 to 12 days. Uninduced cells were used as a control. The DNA content was analysed by flow cytometry, after propidium iodide staining of the nuclei.

	Time after treatment with 2 μg/ml doxycycline				
Clone/cell-cycle phase	Control	+24 h	+72 h	+6 days	+12 days
4B1					
Sub-G_1	13.0	7.9	9.7	6.7	6.7
G_1	43.1	52.7	70.2	66.1	67.8
S	27.9	23.8	12.4	16.5	11.4
G_2/M	16.3	15.6	7.7	10.6	14.5
4B3					
Sub-G_1	7.3	8.3	9.5	8.0	6.9
G_1	42.2	53.1	59.3	67.3	72.7
S	34.3	24.4	17.6	15.7	12.9
G_2/M	16.2	14.4	13.6	9.0	7.5
4B6					
Sub-G_1	10.7	12.0	5.9	6.6	4.1
G_1	44.3	46.4	80.2	75.5	82.9
S	27.3	24.5	9.3	9.4	5.5
G_2/M	18.0	17.1	4.8	8.6	7.2

differences in the sub-G_1 DNA content were observed (Table 1). No effects of doxycycline, on its own, were detected in the parental rtTA-expressing cells (see the Experimental section; results not shown). Finally, cells synchronized at the G_1/S boundary by aphidicolin treatment and then released from the block in the presence or in the absence of doxycycline showed no effects due to Bcl-2 in the S- or G_2/M-phases (results not shown).

These results demonstrate that induction of Bcl-2 expression perturbs the cell cycle of H1299 cells, and this effect is directed towards the G_1-phase. Such an alteration of the cell cycle results in reduced proliferative ability of the carcinoma cells.

Induction of a senescent-like phenotype

Since constitutive overexpression of Bcl-2 in HEC1B cells results in a senescent-like phenotype, we investigated the effects of long-term induction of Bcl-2 in H1299 clones. Uninduced 4B1, 4B3 and 4B6 cells are characterized by an epithelial, cobblestone-like appearance, similar to the parental H1299 cell line (Figure 3A). Short-term induction of Bcl-2 in H1299 clones had no main effect on cellular morphology. However, after long-term induction, clusters of enlarged and markedly flat cells were detected in all clones (Figure 3B). These morphological alterations were apparent by day 6 and were very pronounced by day 9 of doxycycline treatment. In addition, long-term induced cells exhibited increased SA-β-gal staining, as compared with control, uninduced cells (Figure 3C). The expression of SA-β-gal was detected by day 6 of doxycycline treatment. By day 9, approx. 30–40 % of the cells were positive for SA-β-gal staining (Figure 3D).

To substantiate further these observations, we analysed two additional clones with inducible Bcl-2 expression. These clones also demonstrated high induction of SA-β-gal upon doxycycline treatment (Figure 3D). These results show that in a large fraction of H1299 cells, Bcl-2 overexpression induces a permanent cell-cycle arrest with the features of premature senescence.

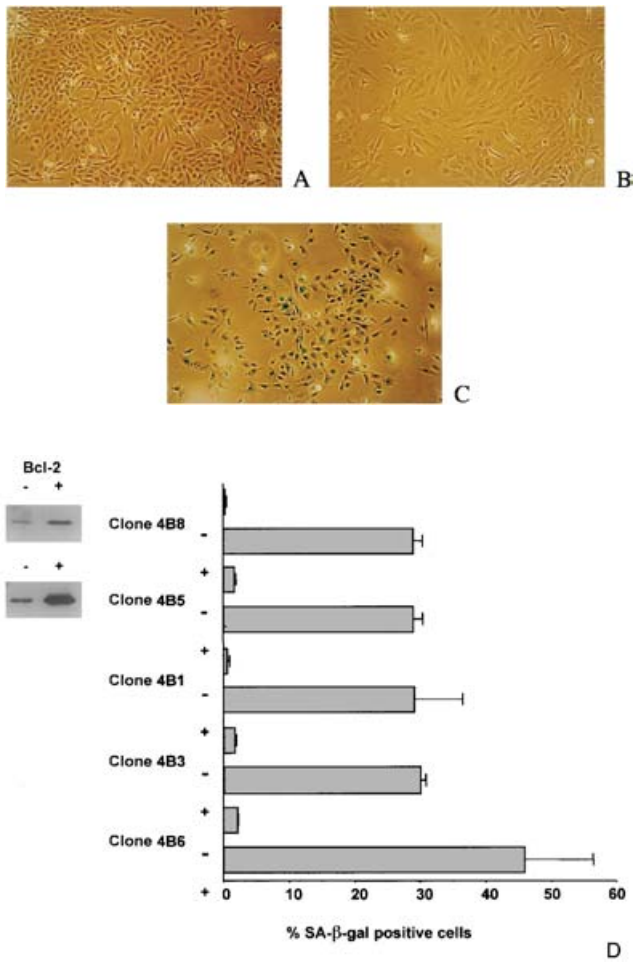

Figure 3 Morphological alterations and SA-β-gal staining in H1299 clones

Triplicate samples of 4B3 cells were plated at 2×10^3 cells per 60 mm dish. Cells were incubated with or without doxycycline (2 μg/ml) for 9 days. Cells were photographed using a Nikon TMS microscope at 100 $\times$ magnification: (**A**) control, uninduced cells; (**B**) morphological alterations in Bcl-2-induced cells; and (**C**) cells stained to detect SA-β-gal activity. (**D**) Triplicate samples of inducible cells were plated at 2×10^3 cells per 60 mm dish. Cells were incubated with (+) or without (−) doxycycline (2 μg/ml) for 9 days. Cells were stained to detect SA-β-gal activity; the percentage of cells positive for SA-β-gal was determined by counting of three random fields under a bright-field microscope. For each independent determination a minimum of 200 cells were counted. The level of Bcl-2 protein in 4B5 and 4B8 clones at 48 h induction is shown.

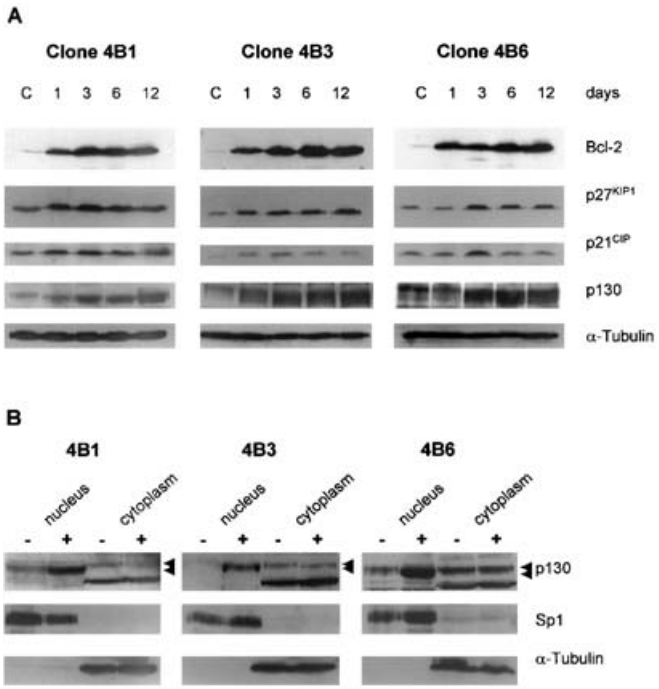

Figure 4 Effects of Bcl-2 overexpression on cell-cycle regulators (A) and subcellular localization of p130 (B)

(**A**) Cells were treated with 2 μg/ml doxycycline for 1 to 12 days and protein expression was detected by Western blotting. Extracts from uninduced cells were used as a control (lanes labelled 'C'). The same filters were stripped and re-probed with anti-α-tubulin antibodies as a loading control. Inducible clones were treated with 2 μg/ml doxycycline for 72 h and subjected to fractionation. Equal amounts of cytoplasmic and nuclear extracts (50 μg) were separated by SDS/PAGE and blotted with p130 antibodies. Arrowheads indicate hyperphosphorylated and hypophosphorylated isoforms of p130. The same filters were stripped and re-probed with anti-α-tubulin antibodies, as a cytoplasmic marker, and anti-Sp1antibodies as a nuclear marker.

The expression pattern of cell-cycle regulatory proteins following the induction of Bcl-2 expression

We examined the protein levels of cell-cycle regulators, following induction of Bcl-2 in H1299 cells. In particular, we analysed those shown to have a critical role in Bcl-2-dependent cell-cycle arrest in immortalized 3T3 murine fibroblasts [11]. In line with this data, induction of Bcl-2 led to an increase in the amount of $p27^{KIP1}$ protein in all clones (Figure 4A), whereas the levels of expression of the other members of the CIP/KIP family, $p21^{CIP1}$ and $p57^{KIP2}$, were unchanged (Figure 4A, and results not shown).

Both $p27^{KIP1}$ and the pRb-related protein p130 have been reported to be essential mediators of the Bcl-2 cell-cycle inhibitory effect [11]. Therefore we also examined the expression of the p130 pocket protein. Asynchronously growing control cells displayed low amounts of the hyperphosphorylated, slowly migrating form of p130. Following induction, as the cells begin to exit the cell cycle to a resting state, the faster migrating, hypophosphorylated forms of p130 accumulated (Figure 4A). p130 protein preferentially interacts with the E2F4 transcription factor, hence the increase in the amount of p130 would lead to the formation of E2F4–p130 complexes in Bcl-2-induced clones. As expected, p130 was co-immunoprecipitated with E2F4 in all clones tested (results not shown). The activity of both p130 and E2F4 is regulated by their subcellular localization. In quiescent cells, E2F4 is nuclear, whereas it shuttles out of the nucleus during S-phase [31]. Similarly, recent data have shown that a cell-cycle-dependent nucleocytoplasmic shuttling contributes to the regulation of p130 activity [32]. Therefore we analysed the localization of p130 and E2F4 in Bcl-2-overexpressing cells by fractionation experiments. Fractionation was verified by Western blotting using α-tubulin as a cytoplasmic marker and Sp1 as a nuclear marker (Figure 4B). As previously shown, upon Bcl-2 induction a dramatic increase in p130 levels was detected. Interestingly, whereas hyperphosphorylated, inactive p130 remained in the cytoplasmic fraction, the hypophosphorylated forms of p130 accumulated in the nucleus. In contrast, no variation in the subcellular localization of E2F4 was observed in Bcl-2-overexpressing cells, as compared with controls (results not shown).

Although an ability of Bcl-2 to influence the phosphorylation status of pRb has been described in other cellular systems [33], little variation in the pRb phosphorylation pattern was observed in H1299 clones after induction (results not shown). Furthermore, antibodies specific for the phosphorylated state of Ser^{807} and Ser^{795} in pRb did not detect significant alterations (results not shown). To

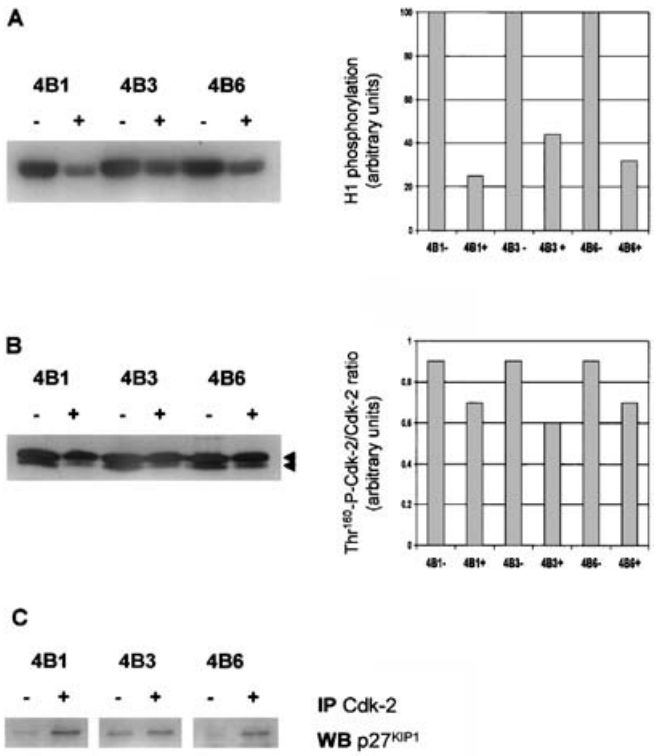

Figure 5 Modulation of Cdk2 kinase activity in Bcl-2-overexpressing cells

Inducible clones were treated with 2 μg/ml doxycycline for 48 h and the ability of immunoprecipitated Cdk2 to phosphorylate histone H1 was assessed by kinase assay. Uninduced cells (lanes labelled '–') were used as controls. The same protein extracts were used to monitor protein expression. (**A**) Histone H1 phosphorylation. Autoradiograms from the kinase assay were scanned by densitometry and associated quantitative data are shown in the histogram. (**B**) Cdk2 protein levels. Immunoblots were scanned by densitometry, and quantitative data are shown in the histogram. (**C**) Cdk2–cyclin complexes were immunoprecipitated from total cell lysates with anti-Cdk2 antibodies. The immunoprecipitates were fractionated on SDS/PAGE and immunoblotted for $p27^{KIP1}$.

investigate further a potential role of pRb in the Bcl-2-dependent cell-cycle arrest, the interaction between E2F1 and pRb upon Bcl-2 induction was analysed by co-immunoprecipitation. No reproducible variations in the amount of E2F1–pRb complexes were detected (results not shown).

These data indicate that overexpression of Bcl-2 in human carcinoma cells results in the accumulation of $p27^{KIP1}$ and p130, which are known to act in anti-proliferative pathways.

Modulation of Cdk2 activity by Bcl-2

Since Bcl-2 overexpression in H1299 cells results in rapid induction of two Cdk2 inhibitors, $p27^{KIP1}$ and p130, we next analysed the activity of Cdk2 in induced cells using histone H1 as the substrate. As shown in Figure 5(A), treatment with doxycycline led to a substantial decrease in Cdk2 activity in all clones. Neither the level of expression of cyclin E (results not shown) nor the total amount of Cdk2 (Figure 5B) was appreciably affected by Bcl-2 overexpression. Anti-Cdk2 antibodies detect two different isoforms of the kinase: one with higher mobility, which has been reported to be the CAK-Thr^{160}-phosphorylated, active form, and the slower isoform, which corresponds to unphosphorylated, inactive Cdk2 [34]. A preferential accumulation of the hypophosphorylated, inactive form of Cdk2 was detected after Bcl-2 induction (Figure 5B). No changes in the amounts of Cdk4, Cdk6, cyclin D1 and cyclin D3 were observed (results not shown). These experiments suggest that the anti-proliferative activity of Bcl-2 is mediated by inhibition of Cdk2.

Increased levels of $p27^{KIP1}$ trigger G_1 cell-cycle arrest through binding and repression of Cdk2–cyclin complexes [35]. Moreover, the pRb-related protein p130 contains both a high-affinity cyclin-binding site and a Cdk2 inhibitory domain. These sequences enable p130 to directly bind and inhibit cyclin E– and cyclin A–Cdk2 complexes *in vivo* [36]. To investigate the relative contribution of these two inhibitors to Cdk2 regulation, we immunoprecipitated Cdk2, and the presence of either associated $p27^{KIP1}$ or p130 was detected by immunoblotting. After induction of Bcl-2, an increase in $p27^{KIP1}$ co-immunoprecipitated with Cdk2 was detected in all clones (Figure 5C). In contrast, no Cdk2-associated p130 was detected in the induced cells (results not shown). These data suggest a critical role for $p27^{KIP1}$ in Bcl-2-dependent cell-cycle arrest in H1299 cells. To substantiate further a role for $p27^{KIP1}$ in the Bcl-2-dependent senescence programme, we investigated the effects of its constitutive overexpression in H1299 clones. Inducible clones were transfected with a HA-tagged $p27^{KIP1}$ or a control vector and analysed after 16 days of puromycin selection. Expression of transfected $p27^{KIP1}$ was assessed by Western blotting (results not shown). As described in other cell systems [37,38], constitutive overexpression of $p27^{KIP1}$ resulted in the acquisition of a senescent-like phenotype in about 60 % of transfected cells (results not shown).

Inactivation of Cdk2 acts downstream of Bcl-2 to induce senescence

The results shown suggest a model in which Bcl-2, via Cdk2 inactivation, induces a premature senescent phenotype in carcinoma cells. To test this hypothesis and to dissect the inhibitory signal downstream of Bcl-2, we decided to directly modulate Cdk2 activity. We generated a tetracycline-inducible construct encoding dn-K2 [28]. Several clones were isolated, and the induction of dn-K2 was analysed by Western blotting using anti-Cdk2 antibodies. Figure 6(A) shows the induction of dn-K2 protein in clones 11, 19 and 20, treated with doxycycline for 48 h. To assess the ability of inducible dn-K2 to inhibit endogenous Cdk2 kinase, we analysed the activity of Cdk2 in induced cells using histone H1 as the substrate. As shown in Figure 6(A), treatment with doxycycline led to a substantial decrease in Cdk2 activity in all clones. It is interesting to note that the extent of Cdk2 inhibition observed upon dn-K2 induction was comparable with those obtained by overexpressing Bcl-2 (compare quantitative data in Figure 6A with those in Figure 5A). Next, we evaluated the effect of dn-K2 induction on the cell cycle. Clones 11, 19 and 20 were incubated with or without doxycycline for 48 h and then analysed by flow cytometry. Induction of dn-K2 results in the accumulation of the cells in the G_1-phase of the cell cycle and a reduction in the number of cycling cells (S-phase; Figure 6A).

Cdk2 has a critical role in the phosphorylation-dependent inactivation of p130, and Cdk2-mediated phosphorylation results in dissociation of the E2F4–p130 complexes. Hence we analysed the phosphorylation pattern of p130 in dn-K2-expressing cells. Indeed, induction of dn-K2 was accompanied by the accumulation of hypophosphorylated, active p130 (results not shown). Finally, since phosphorylation of $p27^{KIP1}$ by Cdk2 targets $p27^{KIP1}$ for proteasomal degradation, we analysed $p27^{KIP1}$ protein levels in induced and uninduced control cells. No changes in the amount of $p27^{KIP1}$ were detected by Western blot in clones 11, 19 and 20 upon dn-K2 induction (Figure 6A).

We examined the ability of dn-K2 to induce a senescent-like phenotype in H1299 cells. Cells were incubated with or without doxycycline for 7 days and then stained for SA-β-gal.

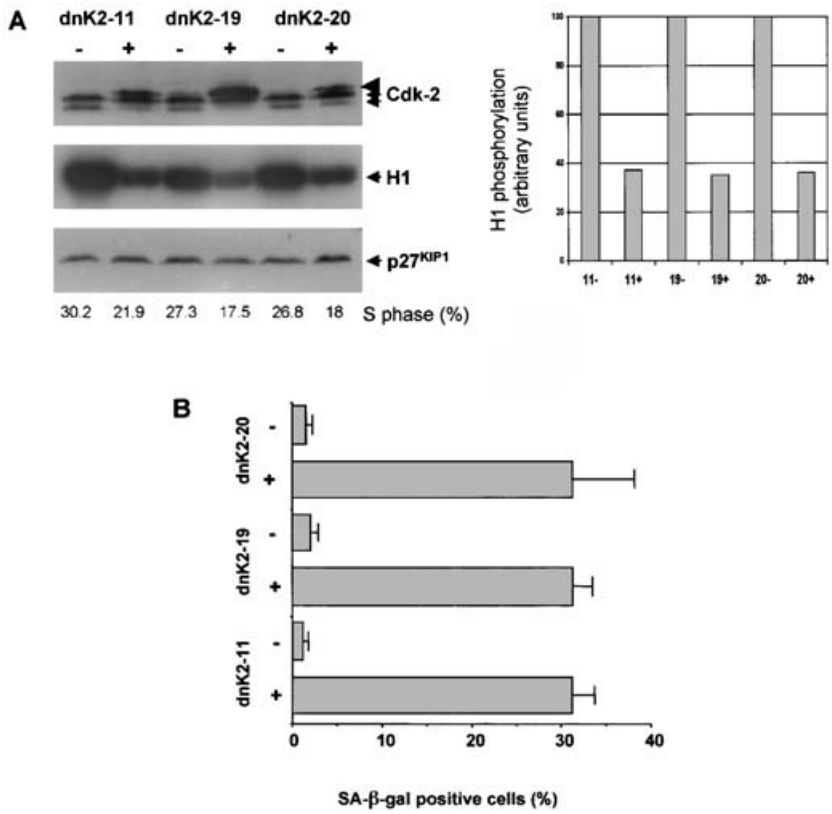

Figure 6 Effects of dn-K2 expression in H1299-inducible clones

(**A**) Cells were treated with 2 μg/ml doxycycline for 48 h and protein expression was monitored by Western blot analysis. Extracts from uninduced cells were used as controls (lanes labelled with '–'). Filters were immunodetected using anti-Cdk2 antibodies. Small arrows indicate the endogenous isoforms of the kinase; the large arrow indicates the position of the ectopic, HA-tagged dn-K2. The same filter was stripped and re-probed with anti-p27^{KIP1} antibodies. The ability of immunoprecipitated Cdk2 to phosphorylate histone H1 was assessed by kinase assay. Autoradiograms from kinase assay were scanned by densitometry and quantitative data are shown in the histogram. The percentages of S-phase cycling cells, following dn-K2 induction, are shown. (**B**) Triplicate samples of clones 11, 19 and 20 cells were plated at 2×10^3 cells per 60 mm dish. Cells were incubated with or without doxycycline (2 μg/ml) for 7 days. Cells were stained to detect SA-β-gal activity; the percentage of cells positive for SA-β-gal was determined by the counting of three random fields under a bright-field microscope. For each independent determination a minimum of 200 cells were counted. Data represent the average and S.D. from three independent observations.

Overexpression of dn-K2 rapidly induced positive staining for SA-β-gal in all clones (Figure 6B). In addition, the majority of induced cells assumed an enlarged and flat morphology. The SA-β-gal positive cells induced by dn-K2 assumed a rounded, less elongated morphology as compared with Bcl-2-induced senescent cells. Distinct phenotypic alterations in response to different senescence signals have also been observed in other cell systems [37].

These data suggest that the p27^{KIP1}-dependent inhibition of Cdk2, downstream of Bcl-2, results in the activation of a premature senescence programme in H1299 cells. To confirm a causative role for p27^{KIP1} in this programme, we sought to interfere with the Bcl-2-dependent up-regulation of p27^{KIP1}.

p27^{KIP1} is required for the maintenance of the senescent phenotype

In order to modulate p27^{KIP1} protein levels in Bcl-2-inducible clones, we decided to use antisense oligos, previously shown to specifically down-regulate p27^{KIP1} in other cell lines [37]. Clones 4B3 and 4B6 were selected for these experiments. The ability of the antisense oligo to down-regulate p27^{KIP1} in H1299 cells was assessed by Western blotting. As shown in Figure 7(A), treatment with 2 μM p27^{KIP1} antisense oligo resulted in substantial reduction of p27^{KIP1} protein following Bcl-2 induction. The effect was specific, since p21^{CIP1} protein levels were not affected. Treatment with scrambled, control oligo affected neither p27^{KIP1} nor p21^{CIP1} proteins (Figure 7A). In addition, increased levels of p130 protein, in Bcl-2-overexpressing cells, were not affected by treatment with p27^{KIP1} antisense oligo (Figure 7A).

The effect of antisense oligo on Bcl-2-dependent cell-cycle arrest was first analysed. 4B3 and 4B6 clones were induced with doxycycline for 48 h in the presence of p27^{KIP1} antisense or scrambled control oligo. Flow-cytometric analyses show that the addition of scrambled oligo did not affect the ability of Bcl-2 to arrest the cells in G_1-phase of the cell cycle; in contrast, p27^{KIP1} antisense oligo resulted in increased fraction of cycling (S-phase) cells (results not shown). These results suggest a critical role of p27^{KIP1} in the maintenance of Bcl-2-dependent cell-cycle arrest in our clones. Therefore we next analysed the ability of antisense oligo to counteract the Bcl-2-dependent inhibition of Cdk2. Inducible clones were treated with doxycycline for 48 h in the presence of p27^{KIP1} antisense or scrambled control oligo, and the activity of Cdk2 was measured using histone H1 as substrate. As expected, treatment with doxycycline led to a decrease in Cdk2 activity in scrambled oligo-treated cells (Figure 7B); treatment with antisense p27^{KIP1} substantially increased Cdk2 activity, restoring Cdk2 activity in Bcl-2-overexpressing cells as compared with controls (Figure 7B).

Next, we used this antisense approach to analyse the effect of p27 down-regulation on Bcl-2-dependent senescence. Cells were

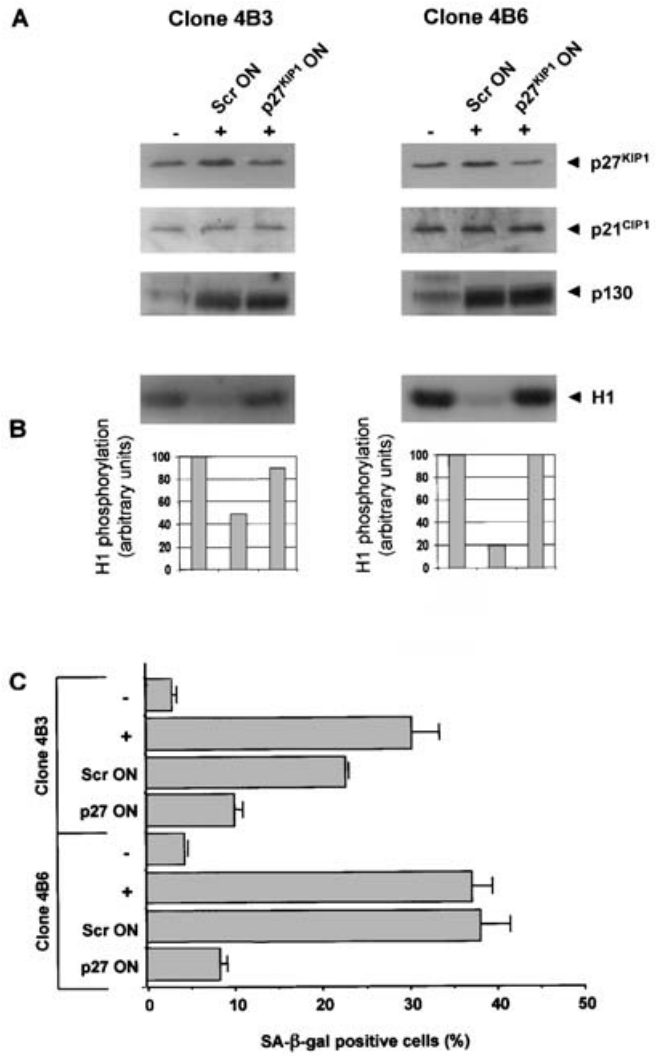

Figure 7 Effects of p27^{KIP1} antisense oligo on Bcl-2-induced senescence

(**A**) Cells were treated either with 2 μM p27^{KIP1} antisense oligos (p27^{KIP1} ON) or with 2 μM scrambled oligos (Scr ON), in the presence of doxycycline (+) for 48 h. Protein expression was detected by Western blotting. Extracts from untreated cells (lanes labelled '−') were used as a control. The same filters were stripped and re-probed with p21^{CIP1} antibodies. For p130 protein analyses, cells were treated for 96 h. (**B**) Cells were treated either with 2 μM p27^{KIP1} antisense oligos (p27^{KIP1} ON) or with 2 μM scrambled oligos (Scr ON), in the presence of doxycycline (+) for 48 h, and the ability of immunoprecipitated Cdk2 to phosphorylate histone H1 was assessed by kinase assay. Uninduced cells (lanes labelled '−') were used as controls. Autoradiograms from the kinase assay were scanned by densitometry, and quantitative data are show in the histogram. (**C**) Triplicate samples of 4B3 and 4B6 cells were plated at 2×10^3 cells per 60 mm dish. Cells were incubated with or without doxycycline (2 μg/ml) for 8 days. Doxycycline-induced cells were subsequently treated either with 2 μM p27^{KIP1} antisense oligos (p27 ON) or with 2 μM scrambled oligos (Scr ON). After 48 h, cells were stained to detect SA-β-gal activity. The percentage of cells positive for SA-β-gal was determined by counting three random fields under a bright-field microscope. For each independent determination a minimum of 200 cells were counted.

induced with doxycycline for 8 days and then incubated with antisense p27^{KIP1} or scrambled oligo, for an additional 48 h. As previously described, long-term, constitutive overexpression of Bcl-2 resulted in increased expression of SA-β-gal in both clones (Figure 7C). p27^{KIP1} antisense oligo substantially decreased the amount of SA-β-gal-positive cells in both clones (Figure 7C; p27 ON), whereas scrambled oligo had no significant effects (Figure 7C; Scr ON). The phenotypic alterations induced by

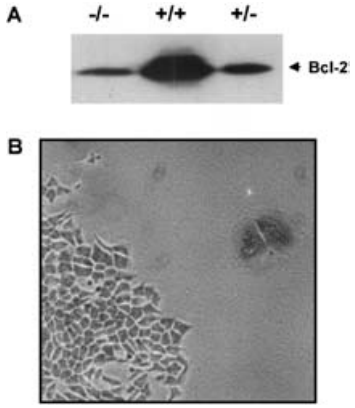

Figure 8 Protein extracts used to monitor Bcl-2 expression (A), and morphological alterations in non-proliferating cells as compared with proliferating, colony-forming cells (B)

Colonies were stained with Methylene Blue.

Bcl-2 (enlarged and flat morphology and reduced saturation density) were retained in most p27^{KIP1} antisense oligo-treated cells, which were negative for SA-β-gal expression. This observation suggests that the decrease in SA-β-gal-positive cells is not simply related to the outgrowth of cells rescued by the cell-cycle arrest, but is determined by the ability of p27^{KIP1} antisense oligo to specifically antagonize the expression of senescence markers. These results indicate that p27^{KIP1} has a key role as transducer of the Bcl-2-dependent senescence signalling pathway.

Permanent morphological alterations in Bcl-2-overexpressing cells

The Bcl-2 inducible clones treated with the p27^{KIP1} antisense oligos retained a flat and enlarged morphology, even though they had a massively reduced SA-β-gal expression. This observation suggests an irreversibility of the senescent-like phenotype induced by Bcl-2. To investigate further whether the morphological alterations in Bcl-2-overexpressing cells are transient or irreversible, we examined the ability of Bcl-2-overexpressing cells to recover a normal morphology upon doxycycline removal, in colony-forming assays. Cells were induced with doxycycline for 7 days and then allowed to grow in normal media for an additional 7 days (Figure 8A; +/− cells). Control cells were continuously treated with doxycycline for 14 days (+/+ cells). Induction of Bcl-2 protein was assessed by Western blotting in these cells. As expected, continuous treatment with doxycycline induced the accumulation of Bcl-2 (Figure 8A; +/+ cells) and removal of doxycycline resulted in a decrease in Bcl-2, which returned to basal levels in +/− cells, as compared with uninduced cells (Figure 8A; compare +/− cells with −/− cells).

Two types of cells could be detected in the +/− dishes: cells with a normal morphology and cells with a large and flat appearance. Significantly, the former gave rise to large colonies, while the ones with a senescent-like phenotype did not proliferate over 7 days of doxycycline removal (Figure 8B). These results show that Bcl-2 can induce a permanent morphological alteration and growth inhibition in a significant subset of H1299 cells.

DISCUSSION

Members of the Bcl-2 family have been originally identified as critical regulators of the apoptotic process. However, these regulators of programmed cell death have also been shown to modulate cell-cycle progression. In particular, overexpression of

the anti-apoptotic members of the Bcl-2 family in normal cells reduces the fraction of proliferating cells, favouring a quiescent over a cycling state [5,6,11]. Overexpression of Bcl-2 in normal, quiescent cells retards entry into the cell cycle, but does not affect their rates of proliferation once in cycle [7,8]. The observation that Bcl-2 exhibited a strong anti-mitotic activity in several solid-tumour-derived cell lines [7,12] suggested a role for Bcl-2 in the control of cell proliferation. The molecular mechanism by which Bcl-2 could severely hamper the growth of carcinoma cells has remained largely unknown. In the present study, we describe the ability of Bcl-2 to induce a senescent-like phenotype in human endometrial carcinoma cells, and then we analysed the anti-proliferative pathway elicited by Bcl-2 in human lung carcinoma cells.

Bcl-2-dependent cell-cycle arrest

We show that Bcl-2 provokes a strong cell-cycle arrest in continuously cycling H1299 cells. The effect of Bcl-2 is directed towards the G_1-phase of the cell cycle and results in a reduced proliferative potential of the carcinoma cells. Two different cell cycle inhibitors are induced in H1299 cells in response to Bcl-2 expression: $p27^{KIP1}$ and p130. This is in agreement with other data [11] which demonstrate an essential role of $p27^{KIP1}$ and p130 as mediators of the effect of Bcl-2 on the cell cycle. Although both $p27^{KIP1}$ and p130 demonstrate an ability to suppress the growth of H1299 cells, they appear to promote cell-cycle arrest through distinct pathways. According to the central role played by CIP/KIP family members in the control of Cdk function, we show that $p27^{KIP1}$ modulates Cdk2 activity through direct binding, as demonstrated by the increase in Cdk2-associated $p27^{KIP1}$. The interaction between $p27^{KIP1}$ and Cdk2 is also consistent with the concomitant loss of Cdk2 kinase activity, which we observe. The ability of antisense $p27^{KIP1}$ oligos to completely restore Cdk2 activity in doxycycline-treated cells support further the idea of a $p27^{KIP1}$-dependent inhibition of Cdk2.

Binding of $p27^{KIP1}$ and p130 to Cdk2 is thought to be mutually exclusive. $p27^{KIP1}$, in fact, has been demonstrated to disrupt the interaction between Cdk2 and p130 [39]. This would explain why our attempts to detect Cdk2-associated p130 in H1299 induced cells were unsuccessful. Therefore we favour the notion that p130 mainly acts as a transcriptional regulator. The accumulation of p130 in the nucleus and the presence of p130–E2F4 complexes in induced cells is consistent with this notion. The ability of p130 to inhibit the transcription of several genes involved in the control of cellular proliferation, such as E2F1–3, cyclin A, cdc25 and cdc2, may have a critical role in both the onset and the durability of a quiescent state.

$p27^{KIP1}$ has been proposed to act either upstream, via Cdk2 inhibition, or in parallel with p130 [11]. The experiments using antisense oligos indicate a $p27^{KIP1}$-independent induction of p130 downstream of Bcl-2 in H1299 cells. The ability of antisense $p27^{KIP1}$ oligos to rescue the cells from the Bcl-2-dependent cell-cycle arrest, in the face of a sustained up-regulation of p130, support further the critical role of $p27^{KIP1}$ as mediator of the anti-mitotic activity of Bcl-2.

These data, as a whole, indicate that the Bcl-2-dependent cell-cycle arrest in H1299 cells is mediated by $p27^{KIP1}$ through direct binding and inhibition of Cdk2.

Induction of a senescent-like phenotype

Constitutive Bcl-2 expression in HEC1B and H1299 cells induces the expression of several distinctive features of cellular senescence, i.e. flat and enlarged morphology (Figures 1, 3 and 8), cytoplasmic vacuolization (Figure 1), expression of SA-β-gal (Figures 1 and 3) and inhibition of proliferation (Figure 2). In addition, senescent cells typically demonstrate increased expression of cyclin-dependent kinase inhibitors and down-regulation of E2F-regulated genes [40], both features displayed by H1299 cells expressing Bcl-2 over a long period.

Although replicative senescence has been originally characterized as a permanent cell-cycle arrest that ensues in normal cells at the end of their life-span [21], it has been subsequently demonstrated that, apart from aging, several different physiological stimuli can trigger senescence in normal human cells [41]. The induction of a senescent-like phenotype has also been demonstrated in tumour-derived cell lines. In particular, activation of a senescence programme in neoplastic cells has been obtained either by reintroduction of critical regulators of replicative senescence [25,37] or by treatment with anti-cancer agents [42]. Most interestingly, expression of several CKI, in both normal and tumour cells [23,24], triggers premature senescence. Two molecular pathways appear to have a critical role in senescence in normal cells: the $p16^{INK4a}$/Rb pathway and the p53/$p21^{Cip1}$ pathway [43]. Neither p53 nor $p16^{INK4a}$ are expressed in H1299 cells. However, overexpression of Bcl-2 in H1299 cells results in the accumulation of $p27^{KIP1}$.

Several of our findings show that $p27^{KIP1}$ acts as a senescence-effector downstream of Bcl-2: $p27^{KIP1}$ protein rapidly accumulates in induced cells; the expression of senescence markers is specifically inhibited by treatment with antisense $p27^{KIP1}$ oligos; and overexpression of $p27^{KIP1}$ induces a senescent-like phenotype. In addition, direct inhibition of Cdk2, by means of a dominant-negative kinase, results in the acquisition of a senescent-like phenotype in a large fraction of H1299 cells. A contribution of $p27^{KIP1}$ to the onset of senescence has previously been suggested in primary mouse fibroblasts treated with phosphoinositide 3-kinase inhibitors [38]. Furthermore, $p27^{KIP1}$ protein appears to be accumulated during senescence in human fibroblasts [44]. These observations, as a whole, indicate a participation of $p27^{KIP1}$ in the control of several different senescence pathways.

Which of the functions of $p27^{KIP1}$ is relevant for the induction of senescence? Apart from the ability to inhibit Cdk2, $p27^{KIP1}$ has also been demonstrated to inhibit the expression of E2F1-dependent genes. In our cell system, p130 appears to modulate E2F factors. Therefore we favour the idea that binding and inhibition of Cdk2 is the first critical action of $p27^{KIP1}$. However, it is interesting to note that different phenotypic alterations characterize senescent cells obtained by Bcl-2 overexpression or dn-K2 overexpression. Therefore we cannot exclude the possibility that additional cell-cycle regulators are activated downstream of Bcl-2.

Role of Bcl-2-dependent cell-cycle regulation and implications for tumorigenesis

Activation of a premature senescence programme in tumour cell lines has been described in response to either ectopic expression of critical mediators of replicative senescence (e.g. pRb, p53 and their downstream effectors), or treatment with anti-neoplastic agents. Is Bcl-2 normally involved in the control of cellular replicative senescence? The accumulation of Bcl-2 protein, which has been reported in several different cell types during senescence, both *in vitro* [45] and *in vivo* [46,47], may support an involvement of Bcl-2 in normal aging processes. However, the activation of a premature senescence programme by Bcl-2, which we describe in two different carcinoma cell lines, has never been observed in normal cells. Hence we believe that the ability of Bcl-2 to induce a senescent-like phenotype is specific for tumour cells.

Accordingly, the cell-cycle inhibitory activity of Bcl-2 is more severe in tumour than in normal cells [7,12]. In this context, it is interesting to note that transformed cells are much more sensitive to Cdk2 inhibition relative to normal cells [48]. Therefore we propose that the capability of Bcl-2 to inhibit Cdk2 only affects the G_1-to-S transition in normal cells [5,11], whereas Bcl-2 expression inhibits proliferation and results in a senescent-like phenotype in carcinoma cells.

The ability of Bcl-2 to modulate Cdk2 may have evolved to counteract its anti-apoptotic activity, as a safeguard mechanism to avoid excessive resistance towards the apoptotic stimuli. Loss of sensitivity to apoptosis, in fact, is one of the hallmarks of tumour cells [22]. In this case, a Bcl-2-dependent senescence programme would be an active mechanism of tumour suppression, in response to an oncogenic stress. Such safeguard mechanisms, however, generally occur in normal primary cells [43]. As previously stated, the cell-cycle inhibitory activity of Bcl-2, in contrast, appears to be much more severe in tumour than in normal cells. Hence, the ability of Bcl-2 to specifically target tumour cells does not support the idea that Bcl-2-induced senescence represents an anti-oncogenic response.

Alternatively, the inhibition of Cdk2 and the consequent cell-cycle arrest may be part of the protective pathway normally activated by Bcl-2. Several observations support this hypothesis. First, the ability to modulate Cdk2 is not limited to the anti-apoptotic members of the Bcl-2 family but is also exhibited by pro-apoptotic proteins; for example, Bax [2]. In addition, even in resting cells, the activation of Cdk2 represents a key event for the induction of programmed cell death [2]; accordingly, activation of cyclin–Cdk2 complexes has been demonstrated during apoptosis in several cell types [49,50]. Hence we favour the hypothesis that the ability to modulate Cdk2 is relevant for the anti-apoptotic function of Bcl-2 in normal cells. Inhibition of Cdk2 in a transformed cellular context results in strong anti-proliferative effects.

The capacity of Bcl-2 to inhibit Cdk2 via $p27^{KIP1}$ up-regulation may well justify its tumour-suppressive activity, which has been demonstrated in several models of *in vivo* carcinogenesis [13–16]. We propose that the observed down-regulation of Bcl-2 during human carcinoma development, as well as the association of Bcl-2 protein with a favourable prognosis and increased patient survival, are related to the ability of Bcl-2 to severely affect the proliferation of carcinoma cells and to induce premature senescence.

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