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Retinoblastoma protein is required for efficient colorectal carcinoma cell apoptosis by histone deacetylase inhibitors in the absence of p21Waf

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Abstract

Colorectal cancer accounts for approximately 10% of all new cancer cases reported worldwide. High dietary fiber intake has been associated with a reduced risk for this type of neoplasia, and much of this effect is ascribed to the histone acetylase (HDAC) inhibitor *n*-butyrate produced in the gastrointestinal tract. Natural chemopreventive and several new synthetic HDAC inhibitors exert multiple effects on tumor cells including the induction of differentiation, cell cycle arrest and apoptosis. Since cancer cells undergo mutational changes, it will be important to understand precisely which pathway gains or losses modulate or compromise HDAC inhibitor efficacy. We have recently documented that *n*-butyrate can provoke apoptosis in human HCT116 colorectal carcinoma cells independently of the p53 tumor suppressor and p21Waf inhibitor. Here, we have developed cell lines on the basis of HCT116 p21−/− cells and HCT116 cells in which the retinoblastoma tumor suppressor protein Rb has been specifically knocked down by antisense expression. The cells were exposed to the DNA-damaging drugs adriamycin (ADR) and etoposide or the HDAC inhibitors *n*-butyrate and trichostatin A (TSA). While the maximal apoptotic response, observed in the absence of p21Waf, was unaffected by the additional knockdown of Rb when cells were treated with ADR or etoposide, the toxicity of the HDAC inhibitors was significantly reduced. This indicates that hyperphosphorylated Rb itself, dissociated from E2F1 transcription factor, can contribute – directly or indirectly – to tumor cell apoptosis provoked by HDAC inhibitors.

Keywords: Histone deacetylase inhibitors; Human colon cancer cells; Retinoblastoma protein; Apoptosis; p53; p21Waf

1. Introduction

Colorectal carcinoma (CRC) is among the leading causes of cancer deaths in the industrialized nations. Recent research has focused on the efficacy of chemopreventive drugs such as the non-steroidal anti-inflammatory drugs (NSAIDs) [1], and chemopreventive natural food compounds, against different stages of CRC. One of the promising cancer-preventive substances is the short-chain fatty acid *n*-butyrate, which is produced mostly by anaerobic bacterial fermentation of dietary fiber in the gastrointestinal tract [2,3]. *n*-Butyrate acts as a potent non-competitive histone deacetylase (HDAC) inhibitor.

HDAC inhibitors have multiple effects on cells. For instance, butyrate has been documented to induce differentiation in human myeloid leukemia cells [4], MCF-7 breast carcinoma cells [5], and colon carcinoma cells [6–10]. A further promising effect of this substance class is the induction of apoptosis. *n*-Butyrate can provoke growth inhibition and death both in vitro and in vivo, for instance, in prostate cancer cells [11,12]. Furthermore, it induces apoptosis in human myeloma cells [13], and growth arrest and apoptosis in colon cancer cells [14,15]. The mechanisms by which *n*-butyrate causes cell cycle arrest and apoptosis are not fully understood but are thought to involve, at least in part, the p21Waf-Rb-E2F pathway.

The gene for the cyclin-dependent kinase inhibitor p21Waf is one major target of the p53 tumor suppressor, and p21Waf overproduction is central for stress-induced, p53-mediated G1 and sustained G2 cell cycle arrests [16,17]. Importantly, p21Waf expression can be provoked through factors other than p53, which is frequently mutated in tumor cells. HDAC inhibitors such as *n*-butyrate have been documented to stimulate p21Waf expression inde-

Abbreviations: CRC, colorectal carcinoma; Rb, retinoblastoma protein; HDAC, histone deacetylase; ADR, adriamycin; TSA, trichostatin A

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pendently of p53, most likely by providing access to binding sites within the p21-promoter for SP1 and SP1-related transcriptional activators [14,18]. We have recently reported that *n*-butyrate can provoke apoptosis in colorectal carcinoma cells lacking p53 or p21Waf, and furthermore, that the absence of p21Waf not only fails to prevent apoptosis but is able to increase the apoptotic response [15]. An increased apoptotic sensitivity to drugs or radiation has also been observed by others with colon cancer cells lacking p21Waf1 [15,16,19,20] or in cancer cells with p21Waf knocked down by antisense RNA expression [21].

The mechanisms by which p21Waf exerts its anti-apoptotic function(s) are unclear. They may include negative effects of the protein on the activation of pro-apoptotic factors such as caspase-3, apoptosis signal-regulating kinase and c-Jun N-terminal kinase [22–24]. However, the requirement – in at least some cases – of the cyclin-dependent kinase inhibitory function of p21Waf for efficient apoptosis-inhibition suggests that the ability of the protein to keep the retinoblastoma protein Rb in its active (hypo-phosphorylated) form, may be important for the anti-apoptotic effect. Along this line, overexpression of Rb in cell lines in vitro has been documented to inhibit apoptosis, and in turn, Rb-knockdown in transgenic animals has been shown to limit tumor growth through apoptotic cell loss (recently reviewed in [25,26]). Combined, this suggests that Rb-deficiency can mimick p21Waf-deficiency with respect to apoptosis sensitization.

How might p21Waf affect apoptosis via Rb? Among the many activities of Rb, perhaps the most important in the context of cell death is its ability to associate with the E2F transcription factors. Importantly, Rb not only neutralizes the transactivation function but also turns E2F1 into a repressor of genes with E2F1 binding sites. Since the Rb/E2F1 repressor complex can dissociate in the absence of p21Waf (leaving E2F1 in its active form) it is conceivable that it may be E2F1 itself that functions as a major mediator of apoptosis in cells lacking functional Rb or p21Waf. Indeed, a large body of evidence implicates E2F1 in apoptosis (reviewed in [27]). On the other hand, E2F-released Rb itself might be a mediator of apoptosis. For instance, Rb is able to form a pro-apoptotic trimeric complex together with p53 and the E3 ubiquitin ligase Mdm-2 [28]. To gain further insight into the role of Rb in drug-induced apoptosis, we have made use of a unique system of pre-existing knockout cell lines [16,17] and newly created knockdown cell lines to ask whether absence of p21Waf and absence of pRb are functionally equivalent in terms of the apoptotic response to drug treatment. We report here that efficient apoptosis induced by HDAC inhibitors is dependent upon Rb whereas, in contrast, apoptosis following treatment with the DNA-damaging drugs adriamycin (ADR) and etoposide is not.

2. Materials and methods

2.1. Chemicals and antibodies

The drugs ADR (adriamycin, doxorubicin), etoposide, sodium butyrate (Na-butyrate), Trichostatin A (TSA), propidium iodide for DNA content analysis, glutaraldehyde and crystal violet for fixation and colony staining were obtained from Sigma (St. Louis, USA). The β-actin monoclonal antibody AC15 was purchased from Sigma, the Rb monoclonal antibody (G3-245) as well as the p53 (DO-1) and the p21Waf (SX 118) monoclonal antibodies were from Calbiochem (San Diego, USA); the caspase-3 polyclonal antibody (PAb CM1) was obtained from BD PharMingen (San Diego, USA).

2.2. Cell culture and transfection

Human HCT116 colorectal adenocarcinoma cells and the p21−/− derivatives were kindly provided by Bert Vogelstein (Johns Hopkins University, Baltimore, USA). HCT116 cells were cultured in McCoy's 5A, supplemented with 10% fetal bovine serum and grown in a humidified 7% CO_2 atmosphere at 37 °C. In order to develop Rb-knockdown HCT116 cells, cultures were transfected with the plasmids pcDNA3.1/Hygro (Invitrogen, Carlsbad, USA) and pcDNA3.1RbAs, containing full length Rb-cDNA in antisense orientation. The transfected cells were selected in medium supplemented with 400 μg/ml hygromycin (PAA Laboratories, Pasching, Austria), and single cell clones were propagated. Exponentially growing cultures that had been taken out of the selection medium for at least two days were either mock-treated or exposed to drug as outlined in the main text and figure legends. For transfection, Effectene from Qiagen (Hilden, Germany) was used according to the manufacturer's recommendations. pGL2-control plasmid (Promega) and the pGL2(CyclinA-promoter) plasmid were used in transient transfections, and luciferase assays were carried out with the luciferase assay system from Promega (Mannheim, Germany) as specified by the manufacturer. The pGL2(CyclinA-promoter) plasmid was kindly provided by J.M. Blanchard, Institut de Genetique Moleculaire de Montpellier, France.

2.3. Flow cytometry analyses of DNA content and apoptosis

One day before drug treatment, 7.5×10^4 cells were seeded in 12-well dishes. After the indicated times, the cells on the dishes were harvested by trypsinization and combined with the cells floating in the medium, fixed, and stained with propidium iodide as described before [15]. DNA fluorescence was measured with the Beckton Dickinson FACScan (Bedford, USA), and the data were analyzed with the CellQuest software from Beckton. For FACS analysis of transiently transfected cells, 1×10^5

cells were seeded in 6-well dishes and transfected according to the manufacturer's recommendations. After 72 h, cells were either mock-treated or treated with drugs. Drugs were used as indicated in the figure legends. Statistical analysis (Student's *t*-test) was performed with the SigmaPlot software from SPSS (Chicago, USA).

2.4. Colony formation assays

Live cells (10^3), counted with a CASY cell counter, were seeded onto 10 cm dishes and grown for 24 h. The cultures were then incubated in the presence of *n*-butyrate (0.2 and 0.5 mM) or TSA (75 nM), or were mock-treated for 10 days. Colonies were washed with PBS several times, fixed with 1.25% glutaraldehyde for 20 min at RT, washed again, and stained with 1% crystal violet in PBS for 1 h at RT.

2.5. Immunoblot analysis

Cells growing on 10 cm dishes were lysed in 150 μl of a lysis buffer heated to 85 °C and containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS and 20 % glycerol. Samples containing 15 μg of total cellular protein were subjected to 8% SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, USA). Signals were detected upon overnight incubation of the membranes with anti-Rb (1:500), anti-p53 (1:2000), anti p21Waf (1:1000), anti-β-actin (1:5000) or anti-caspase 3 (1:2000) antibodies followed by a final incubation with a peroxidase-conjugated secondary anti-mouse antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, USA), performed as specified by the supplier. Signals were quantified by the AIDA 2.31 image analysis software from Raytest (Straubenhardt, Germany).

3. Results

The poorly differentiated, growth factor-insensitive human HCT116 colorectal carcinoma cell line exhibits the microsatellite instability (MIN) phenotype that is associated with a deficiency for hMLH1. This protein is the human homologue of the bacterial Mut L protein, which is also an essential factor of DNA mismatch repair. HCT116 cells respond to several forms of stress including DNA damage and transcription inhibition, not least because of the presence of a functional p53-p21Waf-Rb-E2F tumor suppressor pathway. The availability of several derivative cell lines with tumor suppressor, cell cycle regulatory and apoptotic genes knocked out by targeted homologous recombination [16,17] has made these cells widely used for the study of drug action (see, for instance, [1,20,29]). Important insights came from the previous observations that the presence or absence of p53 or p21Waf can profoundly affect the survival of HCT116 cells treated with anti-cancer and chemopreventive agents, and that these effects are dependent on the type of drug. For instance, the chemotherapeutic drugs adriamycin (ADR) and etoposide caused more apoptosis in the absence of p53, and still more cell death in the absence of p21Waf, establishing both proteins as survival factors with a (limited) protective effect [15,20]. In some contrast, the cell death induced by the chemopreventive short-chain fatty acid *n*-butyrate was not affected by the absence of p53, probably because *n*-butyrate was able to induce p21Waf expression in a p53-independent manner [14,15]. In p21Waf knockout cells, however, *n*-butyrate – like ADR and etoposide – was an efficient inducer of apoptosis.

Absence of p21Waf can cause hyperphosphorylation of Rb through the unrestrained activity of cyclin-dependent kinases. Indeed, comparison of Rb from the parental HCT116 cells and the p21−/− derivatives in immunoblot analyses revealed the presence of hyperphosphorylated Rb in the latter (see, for example, Fig. 1a; and data not shown). Hyperphosphorylated Rb dissociates from, and thereby activates E2F1; similarly, E2F1 is active when Rb protein is absent from the cell. To study the consequences of Rb deficiency in a p21−/− background for drug sensitivity, we developed HCT116 p21−/− cell lines stably transfected with either the empty pcDNA3.1/hygro plasmid conferring hygromycin resistance as a control, or with the same plasmid expressing, in addition to the hygromycin resistance gene, the complete human *rb* gene in antisense orientation. Initial attempts of Rb knockdown with the stable expression of *rb* siRNA constructs on the basis of the pSUPER plasmid [30] were unsuccessful and the concept was abandoned. For the following studies, the two new cell lines HCT116 p21−/− RbAs-2 and –3 were employed, which in Western blots produced only 7.9 and 7.2% of the Rb steady state levels present in the HCT116 p21−/− control cells (Fig. 1a). Of note, the p21−/− Rb knockdown cells expressed strongly increased steady state levels of p53 (Fig. 1b, upper panels) although the p14ARF protein which can stabilize p53 in the absence of Rb (i.e., in the presence of functional E2F1) is not expressed in HCT116 cells [31]. Exposure to the HDAC inhibitors *n*-butyrate (2 mM) or TSA (75 nM) increased the p53 levels only slightly above the levels observed in mock-treated cultures, and the Rb levels stayed knocked down in the presence of the drugs (Fig. 1b). Nonetheless, despite the elevated p53 levels, cell proliferation analyses failed to reveal large differences in the population doubling time between the untreated p21−/− control and p21−/− Rb knockdown cultures (in fact, the knockdown cells grew slightly faster; Fig. 1c), and apoptosis assays documented approximately equal basal apoptosis rates in both cultures (see Fig. 2a). This suggested that the strongly accumulated p53 protein was not active, which was underscored by the fact that, inspite of the elevated p53 protein levels in the absence of Rb, there was no transcriptional activation of the p53 target gene *mdm-2* (data not shown).

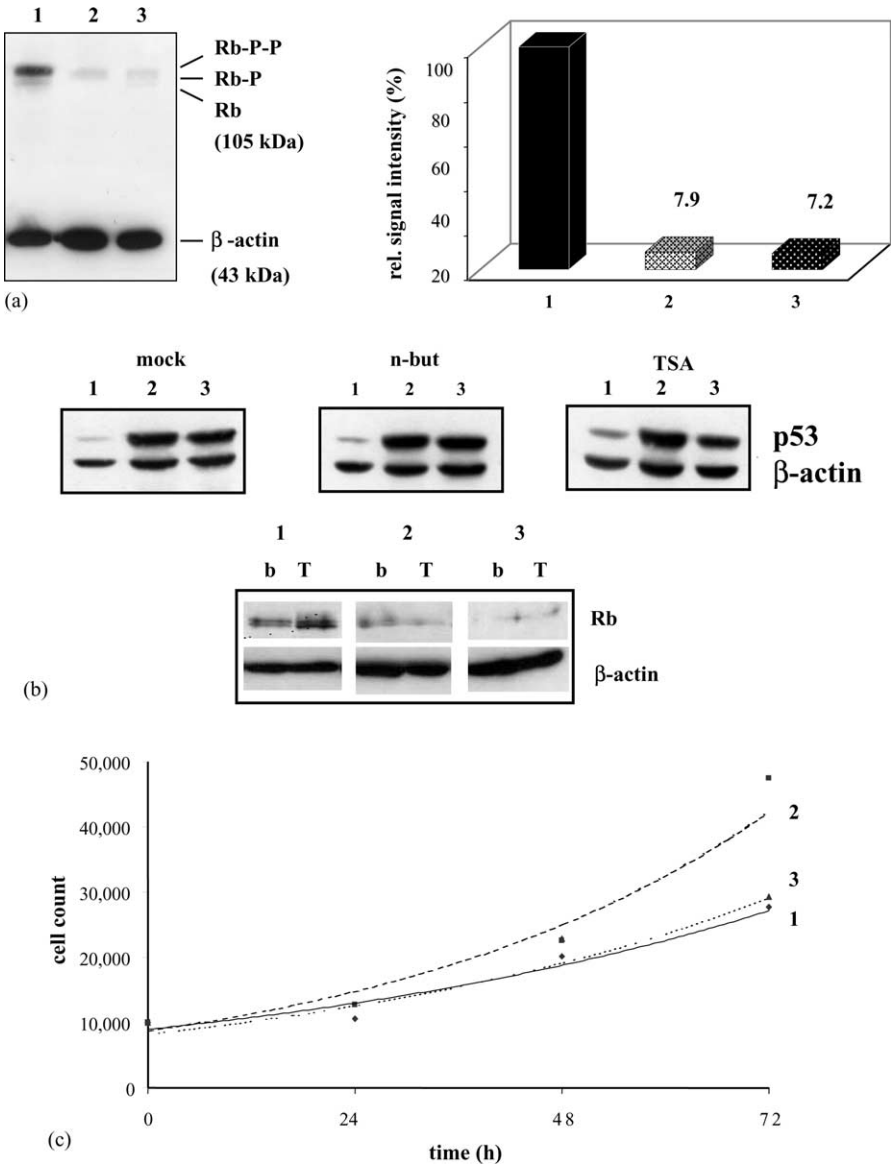

Fig. 1. Development and characterization of the Rb knockdown cell lines HCT116 p21−/− RbAs-2 and RbAs-3. (a) Western blot analysis of HCT116 p21−/− cells stably transfected with control vector (1) or with vector expressing the full-length human *rb* gene in antisense orientation: HCT116 p21−/− RbAs-2 (2); HCT116 p21−/− RbAs-3 (3). Fifteen micrograms of total cell protein was subjected to SDS-PAGE and immunoblot analysis. The monoclonal Rb antibody was used at a dilution of 1:500; the β-actin antibody at 1:5000. The relative signal intensities were determined by laser densitometry. Note the relative strength of the upper as compared to the lower Rb signals generated by hyperphosphorylated Rb (Rb-P-P) in lanes 1–3. (b) Western blot analysis of total cell protein. Cultures were either mock-treated or exposed to *n*-butyrate (*n*-but, b; 2 mM) or TSA (T; 75 nM) for 24 h, and 15 μg of total cell protein was subjected to SDS-PAGE and immunoblot analysis, with the anti-p53 antibody at 1:2000, the anti-Rb antibody at 1:500, and the anti-β-actin antibody at 1:5000. (c) Cell proliferation analysis of the three cell lines. The numbers of cells on 6-well dishes were determined at the indicated times with a CASY cell counter.

When exponentially growing cultures of p21−/− and p21−/− Rb knockdown cells were either mock-treated or exposed to the DNA double strand breaks-inducing drugs ADR (0.34 μM) or etoposide (10 μM), and were analyzed by flow cytometry for cells with a sub-2*n* DNA content indicative of apoptosis, both drugs provoked cell death with equal efficiency, regardless of the presence of Rb (Fig. 2a; $P < 0.001$). This indicated that the apoptosis induced by this kind of insult was not dependent on Rb. By contrast, when the cultures were incubated with *n*-butyrate (2 mM) or TSA (75 nM) and were analyzed at different timepoints, apoptosis was efficiently induced only in the p21−/− cells, while it was clearly inhibited in the p21−/− Rb knockdown cultures (*n*-but: $P < 0.0001$; TSA: $P < 0.01$; Fig. 2a shows the 48 h timepoint). In accord with the flow cytometry analyses, Fig. 2b confirms that exposure of HCT116 cells to the HDAC inhibitors results in the activation of the major apoptosis-executing protease caspase 3, showing that these drugs can provoke apoptosis. The differences in apoptosis induction between the Rb-proficient and deficient cultures were confirmed in colony formation assays. For instance, a concentration of 0.5 mM of *n*-butyrate inhibited the formation of p21−/− colonies completely but allowed the

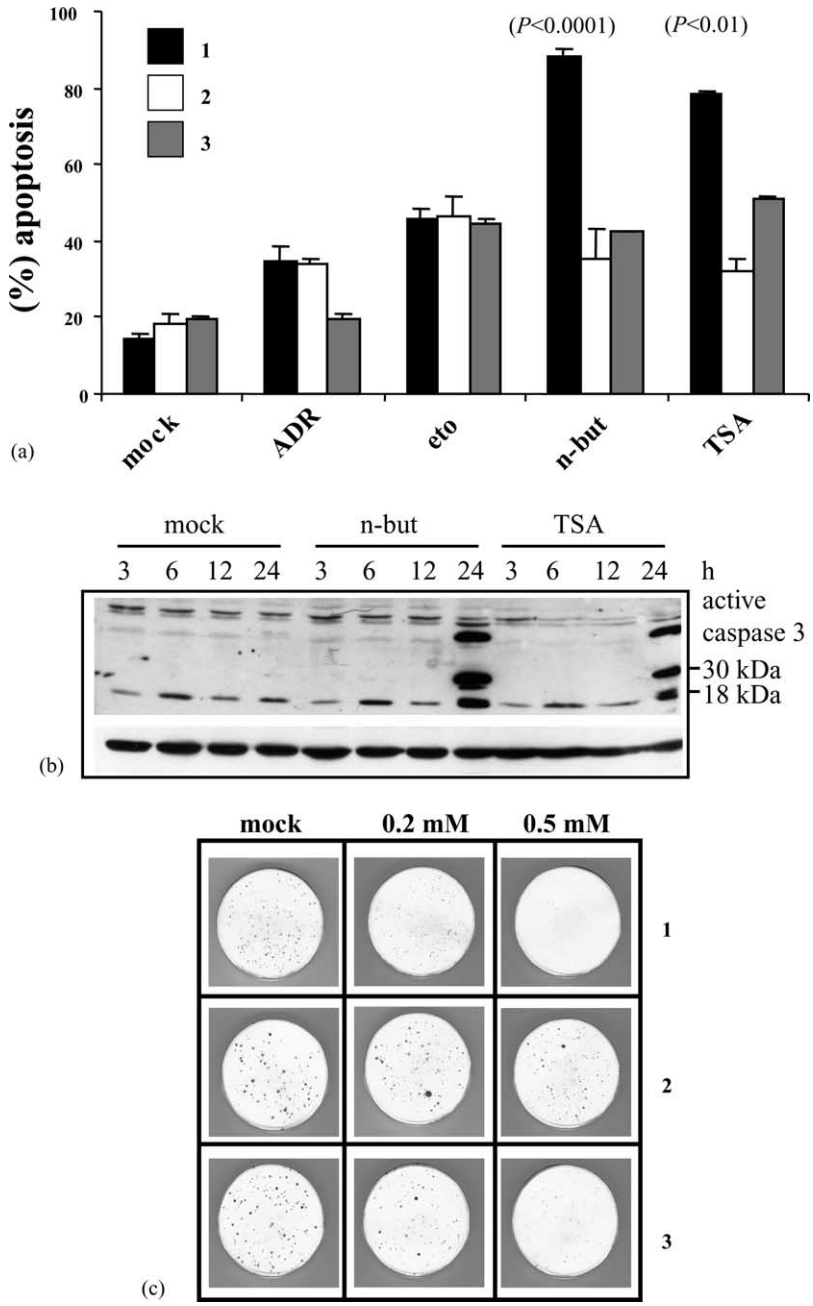

Fig. 2. Effects of chemotherapeutic drugs and HDAC inhibitors on the survival of control and Rb knockdown cells. (a) Exponentially growing cultures of HCT116 p21−/− (1), HCT116 p21−/− RbAs-2 (2); HCT116 p21−/− RbAs-3 (3) cells were either mock-treated or treated with ADR (0.34 μM), etoposide (eto; 10 μM), *n*-butyrate (*n*-but; 2 mM) or TSA (75 nM) and were analysed at 48 h post-treatment, following fixation and propidium iodide staining, for total DNA content by FACS analysis. Cells with a sub-2*n* DNA content were counted as apoptotic. Shown are the mean values + standard deviation for $N = 3–6$ replicates. *P*-values indicating significance of the differences in apoptosis between Rb-proficient and deficient cells are included. (b) Western immunoblot analysis of 15 μg of total cell extracts from HCT116 cultures treated for the indicated times with *n*-butyrate (*n*-but; 2 mM) or TSA (75 nM) and incubated with the polyclonal anti-caspase 3 antibody at 1:2000. (c) Live cells (10^3) of the cell lines designated 1, 2 and 3 in (a) were seeded onto 10 cm dishes and were eventually mock-treated or exposed to the indicated doses of *n*-butyrate for 10 day; the cells were then fixed and the colonies were stained with crystal violet.

formation of p21−/− Rb knockdown colonies, albeit at reduced numbers when compared to mock-treated cultures (Fig. 2c). Similarly, TSA treatment suppressed colony formation in the p21−/− cells more efficiently than in the Rb knockdown cells. Thus, efficient apoptosis induction by HDAC inhibitors is dependent on the presence of Rb in human HCT116 p21−/− colon carcinoma cells.

To study the effect of Rb on the drug-sensitivity of parental, p21Waf-proficient HCT116 cells, we next stably transfected HCT116 cells (containing p21Waf) with either the empty vector or vector expressing the full-length *rb* gene in antisense orientation. On first inspection it turned out that not only were the numbers of stable colonies much lower than in the earlier transfections with HCT116 p21−/− cells, but the obtainable degree of knockdown of Rb was

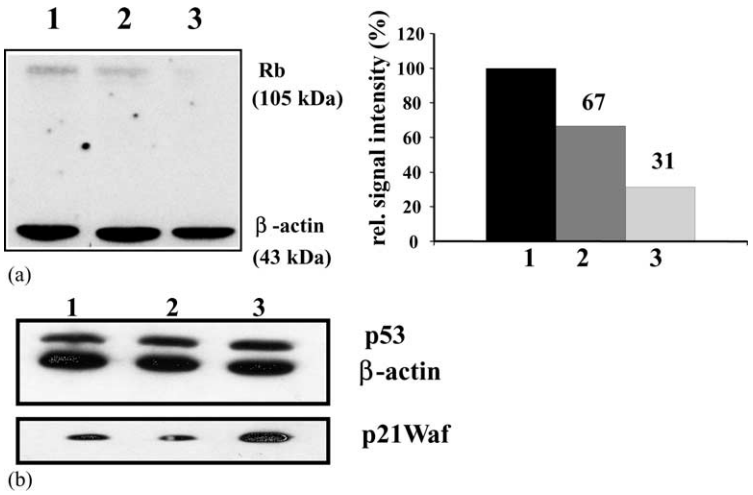

Fig. 3. Development and characterization of the Rb knockdown cell lines HCT116 RbAs-2 and RbAs-14. (a) Western blot analysis of HCT116 cells stably transfected with control vector (1) or with vector expressing the full-length human *rb* gene in antisense orientation: HCT116 RbAs-2 (2); HCT116 RbAs-14 (3). Fifteen micrograms of total cell protein was subjected to SDS-PAGE and immunoblot analysis. The monoclonal Rb antibody was used at a dilution of 1:500; the β-actin antibody at 1:5000. The relative signal intensities were determined by laser densitometry. (b) Western blot analysis of the same cell lines as in (a), with anti-p53 antibody used at a dilution of 1:2000, the β-actin antibody used at 1:5000 and the p21Waf antibody diluted 1:1000.

generally low (Fig. 3a), indicating that a reduction of Rb levels is less well tolerated in the presence of p21Waf than in its absence in these cells. One reason for this may be that overproduced p21Waf can contribute to a G2/M cell cycle arrest independently of Rb [17]. The level of p21Waf was indeed increased in the cell line with the strongest Rb knockdown; p53 levels were not significantly altered (Fig. 3b). Fig. 3a shows that the HCT116 RbAs clones 2 and 14 express Rb to 67 % and 31 % of the parental cells. In a first set of experiments, HCT116, HCT116 RbAs and HCT116 p21−/− cells were treated with ADR and etoposide. Both drugs have been documented to be potent inducers of apoptosis in p21−/− cells [15]. Here, we show that a reduction of Rb expression by one third to two thirds is sufficient to mimick absence of p21Waf with respect to apoptosis induction by ADR and etoposide: cell death is increased in the HCT116 RbAs cultures as well as the p21−/− cultures relative to the parental HCT116 cells (RbAs: $P < 0.05$; p21−/−: $P < 0.001$; Fig. 4a). Thus, absence of p21Waf and knockdown of Rb are functionally equivalent with respect to apoptosis induction by the chemotherapeutic agents ADR and etoposide in these cells, suggesting that active pro-apoptotic E2F1 transcription factor may contribute to this cell death. In contrast, when parental HCT116 cells and HCT116 RbAs 2 cells (with 67% Rb steady state levels relative to the parental cells) were treated with *n*-butyrate or TSA, both cell lines underwent apoptosis, but the Rb knockdown cells did so reproducibly at a slightly reduced level ($P > 0.05$; Fig. 4b), in accord with our previous observation that an almost complete knockdown of Rb in the p21−/− cells inhibits apoptosis provoked by HDAC inhibitors significantly

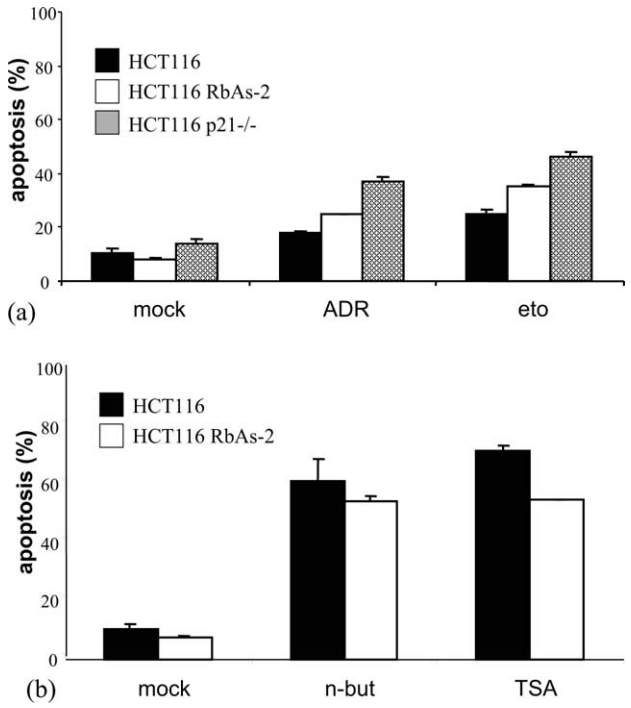

Fig. 4. Cell death induced by chemotherapeutic drugs and HDAC inhibitors. (a) Exponentially growing cultures of HCT116, HCT116 RbAs-2; HCT116 p21−/− cells were either mock-treated or treated with ADR (0.34 μM) or etoposide (eto; 10 μM), and were then analysed at 48 h post-treatment, following fixation and propidium iodide staining, for total DNA content by FACS analysis. Cells with a sub-2*n* DNA content were identified as apoptotic. (b) The same cell lines were again either mock-treated, or exposed to *n*-butyrate (*n*-but; 2 mM) or TSA (75 nM), and were subsequently analyzed by FACS as before. The relative numbers of cells with a sub-2*n* DNA content was used synonymously with the percentage of apoptotic cells. Each value represents the mean values + standard deviation for $N = 3$–6 replicates.

(see Fig. 2). This suggests that efficient colon tumor cell apoptosis by HDAC inhibitors is in part dependent on Rb, regardless of the presence or absence of p21Waf.

The reasons for the remarkable relative resistance of HCT116 p21−/− RbAs cells to HDAC inhibitors are currently unclear. However, cell cycle analyses of the p21−/− Rb knockdown cultures have revealed that these cells differ from the control cell lines in at least one respect. While exponentially growing HCT116 cells and the cells with either a slightly reduced Rb content or lacking p21Waf were predominantly diploid, the p21−/− cells that had, in addition, Rb knocked down were primarily tetraploid in FACS analyses (Fig. 5a, left panel). This was most likely due to endoreduplication, which is known to ensue from the absence of p21Waf (which would be able to put a break on the cell cycle in G2/M even in the absence of Rb) plus an over-activity of E2F1 (which follows the absence of Rb). When the cells were treated with *n*-butyrate, strong peaks produced by apoptotic cells with a sub-2*n* DNA content dominated in the FACS profiles (Fig. 5a, right panel). The cyclin A gene is among the targets of E2F1, and we were indeed able to show, in transient transfections, that a cyclin A promoter in front of a luciferase gene is strongly activated in cells lacking both p21Waf and Rb (Fig. 5b). Thus, while the ploidy differences and E2F1 over-activity seem to have failed to affect the apoptosis provoked by ADR and etoposide, they may contribute to the inhibition of the apoptosis induced by HDAC inhibitors.

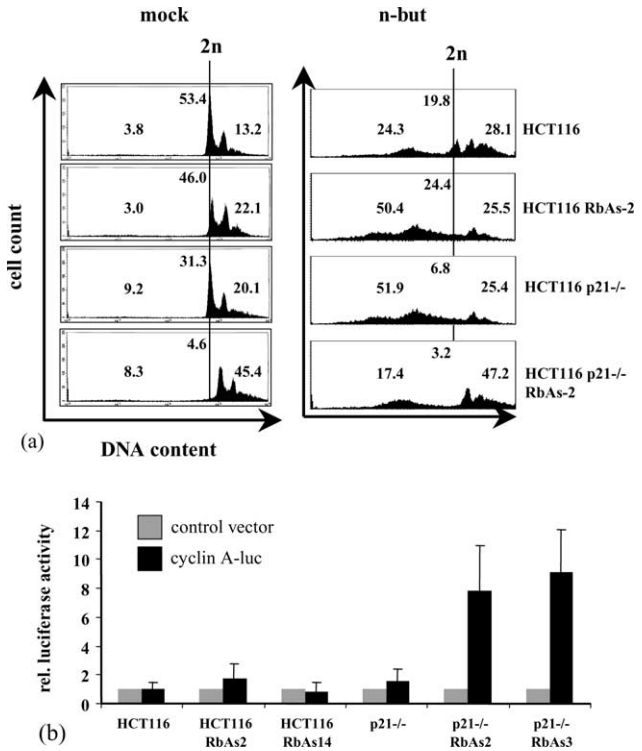

Fig. 5. Polyploidy and activity of the cyclin A promoter indicative of endoreduplication specifically in cells lacking p21Waf plus Rb. (a) FACS profiles of untreated (mock) and *n*-butyrate-treated (*n*-but; 2 mM), exponentially growing cultures of the indicated cell lines at 24 h after treatment. Total cell DNA was stained with propidium iodide; 2*n* designates the position of cells with a 2*n* DNA content. The signals left of 2*n* (cells with a sub-2*n* DNA content) in the right scheme (*n*-but) identify apoptotic cells. The numbers indicate the percentages of sub-2*n*, 2*n* and >4*n* cells, respectively (from left to right). (b) Luciferase activity in total cell extracts from the indicated cell lines. Cells were transiently transfected with a reporter plasmid harboring the E2F1 transcription factor-sensitive human cyclin A promoter upstream of a luciferase reporter gene, and were analyzed at 48 h after transfection. The diagram shows the relative luciferase activity, with the activity following empty vector transfection arbitrarily set as one. Depicted are the mean values + standard deviation for $N = 6$ replicates.

4. Discussion

HDAC inhibitors are a relatively new class of drugs with anti-cancer potential. Some including the short-chain fatty acid butyrate occur as natural chemopreventive substances as the result of bacterial fermentation of dietary fiber in the gastrointestinal tract [2,3]. The limitation of the pharmacological utility of butyrate for other than intestinal neoplasias by its short in vivo half-life of only minutes in plasma [32] has been overcome by the development of stable analogues [33] and new HDAC inhibitors including the hydroxamic acids trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA). The potential advantages of these compounds in comparison with other anti-cancer drugs and chemopreventive agents are several-fold: HDAC inhibitors are effective not only against proliferating cells but also the non-proliferating cells [34] often present in solid tumors [35]. The compounds exhibit cytostatic activities probably primarily through the p53-independent activation of the p21Waf inhibitor of cyclin-dependent kinases that is observed at relatively low drug doses in tumor cells as well as in normal cells [36]. Cytotoxicity may be caused through mitochondria mediated pro-apoptotic activities at higher drug doses that also seem to be mostly p53-independent [11–15,37–39]. However, an early cell death induced by these drugs has been reported to be p53-dependent [40–44]. The p53-independency of much of the drug action is of course important since the p53 tumor suppressor is frequently dysfunctional in tumor cells. Despite of recent advances, the precise mechanisms that underly HDAC inhibitor functions are not fully understood. In particular, the roles of other cell cycle regulators and tumor suppressors such as p21Waf and the retinoblastoma protein Rb – the latter is frequently altered in tumor cells and thus likely to affect HDAC inhibitor efficacy – remain unclear.

Functional Rb is frequently absent in tumor cells, unleashing the pro-proliferative activities of the Rb-inhibited E2F1 transcription factor [26,27]. In contrast to Rb's role in anti-proliferation, its function in cell survival control is complex and seems to be dependent on cell context: free E2F1 may induce apoptosis through p53-dependent and -independent mechanisms (reviewed in [27]), and Rb may thus act anti-apoptotic by inhibiting E2F1. On the

other hand, Rb has been identified as part of a trimeric pro-apoptotic protein complex with Mdm-2 and p53 [28]. Here, we have documented that Rb is necessary for the efficient induction of apoptosis by HDAC inhibitors, at least in HCT116 p21−/− colon carcinoma cells. This may be relevant for the recently developed concept proposing that inhibition of p21Waf combined with conventional chemotherapy will eliminate tumor cells much more efficiently since p21Waf-deficiency can sensitize tumor cells to apoptosis induced by drugs including HDAC inhibitors [15,20]. Loss of Rb might neutralize the potent co-operative anti-tumor effect of HDAC inhibitor treatment and p21Waf inhibition.

The fact that efficient cell death is provoked in the face of p21Waf-deficiency (i.e., Rb hyperphosphorylation and dissociation from E2F1) but not in the absence of Rb suggests that it is hyperphosphorylated Rb itself which, directly or indirectly, affects HDAC inhibitor-provoked apoptosis. In agreement with several previous reports [14,15] it seems unlikely that p53 has a major role in this apoptosis since p53 protein accumulated in the Rb knockdown cells inspite of a significant reduction in apoptosis (see Fig. 1b). Moreover, the accumulated p53 was apparently transcriptionally inactive since no stimulation of transcriptional targets was observed. How then might lack of Rb de-sensitize cells to apoptosis by HDAC inhibitors?

It seems now well established that HDAC inhibitors can initiate apoptosis through several different pathways that nonetheless all primarily execute cell death through activation of the intrinsic mitochondria-mediated pathway. For example, an early p53-dependent and a late, p53-independent form of cell death have been associated with exposure to these compounds [37–44]. Secondly, cell death has been proposed to ensue from the loss of an HDAC inhibitor-sensitive G2 cell cycle checkpoint and the consequential occurrence of aberrant mitosis [36]. Thirdly, HDAC inhibitor-initiated apoptosis has recently been shown to happen even in non-proliferating tumor cells, through as yet unidentified mechanisms [34]. Since the anti-apoptotic effect of Rb-deficiency reported here was not complete in so far as it merely reduced but failed to abrogate apoptosis totally, it seems reasonable to assume that Rb supports only a subset of these apoptotic responses. Through which mechanisms might this be accomplished? It has been shown that tumor cells exposed to HDAC inhibitors usually accumulate with a 4*n* DNA content at the G2 checkpoint [36,45]. If it were precisely these cells that are sensitive to apoptosis, as has indeed been suggested by recent findings [36,45], then exit from the G2 arrest could save these cells (although at the expense of genomic stability). As we have documented in Fig. 5, Rb-deficient HCT116 p21−/− cells were – in contrast to the parental HCT116 p21−/− cells – polyploid, indicative of the overrun of the G2 checkpoint. Consequently, such cells might be less sensitive to HDAC inhibitor-induced apoptosis, as observed.

Several other scenarios are conceivable. Recent observations have indicated that apoptosis by HDAC inhibitors is dependent on the activation and translocation to the mitochondria of the pro-apoptotic Bcl-2 family member Bid [38,40,46]. It is unclear at present whether Rb can affect Bid activation. Finally, previous work has shown that Rb can have a direct pro-apoptotic function in cells through the formation of a trimeric complex with Mdm-2 and p53 that supports the repression of genes by p53 [28]. Among the p53-repressed genes is the one for the anti-apoptotic Bcl-2 protein that has been demonstrated to effectively block apoptosis by HDAC inhibitors (for instance, [40,45]). Future work might aim in particular at determining whether Rb-deficient tumors are less sensitive to these compounds.

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