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Enrichment of non-synchronized cells in the G1, S and G2 phases of the cell cycle for the study of apoptosis

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Abbreviations:
BrdU, 5-bromo-2-deoxyuridine
Casp-3a, activated caspase-3
FACS, fluorescence-activated cell sorter
GFP, green fluorescent protein
H2B, histone H2B
H3P, phosphorylated histone H3
PI, propidium iodide
STS, staurosporine

ABSTRACT

The susceptibility of cells to apoptosis induction is deeply influenced by their position in the cell cycle. Unfortunately, however, current methods for the enrichment of cells in defined phases of the cell cycle are mostly based on the synchronization of cells by agents or conditions that are intrinsically toxic and induce apoptosis on their own. We developed a novel procedure for the purification of cells in distinct phases of the cell cycle. This method is based on the stable transfection of cells with a chimeric protein made up by histone H2B and green fluorescent protein (GFP). Cytofluorometric purification of cells defined by their size and their H2B-GFP-dependent fluorescence (which reflects chromatin and hence DNA content) allowed for the efficient separation of diploid and tetraploid cells in the fluorescence-activated cell sorter (FACS). Moreover, when applied to diploid cells, this method allowed for the enrichment of live, functional cells in the G1, S and G2 phases of the cell cycle. FACS-purified cells were viable and readily resumed the cell cycle upon reculture. While staurosporine was equally toxic for cells in any phase of the cell cycle, camptothecin was particularly toxic for cells in the S phase. Moreover, BAY11-7082, a specific inhibitor of the IKK complex required for NF-κB activation, exhibited a particular cell cycle-specific profile of toxicity (G2 > S > G1). These results delineate a novel procedure for studying the intersection between cell cycle regulation and cell death mechanisms.

1. Introduction

One of the principal hallmarks of apoptosis is DNA fragmentation [1,2]. Progressive DNA degradation leads to accumulation of "subdiploid" or "hypodiploid" cells, which are detected by analyzing the DNA content of the cell population, usually after labeling cells with DNA-intercalating agents [3–5]. This technique is also used as a standard procedure to distinguish cells in the G1, S and G2 phases of the cell cycle. It is without doubt that this technological overlap has boosted the initial interest in the common regulation of cell cycle and cell death. It is well established that many widely used apoptosis inducers act in a cell cycle-specific fashion. Standard examples include topoisomerase inhibitors (that act during

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the S phase) [6], microtubular inhibitors (that act during mitosis) [7] and some DNA damaging agents that induce mitotic catastrophe (that is apoptosis during mitosis) [8]. The most prominent pro-apoptotic transcription factor, p53, is well known to respond to DNA damage (which frequently is detected during the S phase), and many studies have outlined mechanistic links between p53, cell cycle progression and apoptosis [9–11].

Methods for the simultaneous detection of cell cycle-specific alterations and apoptotic parameters have been developed. One example is the combination of the Tunel technique with DNA content analysis, which is convenient for cytofluorometric analyses [5]. Another example is the combined detection of cell cycle-specific proteins (such as cyclins) and apoptotic changes (such as mitochondrial cytochrome *c* release or staining with antibodies recognizing active Bax or mature caspase-3) by means of multi-color immunofluorescence stainings [12,13].

Yet another approach for studying the intersection between cell cycle regulation and cell death mechanisms is the manipulation of the cell cycle, usually by synchronization by agents that prevent DNA synthesis (such as hydroxyurea, aphidicolin or high-dose thymidine) or that inhibit the formation of mitotic spindles (such as spindle poisons including the reversible inhibitor nocodazole), leading to the arrest in precise phases of the cell cycle (early S phase and late G2, for these examples) [14–16]. Unfortunately, these techniques have the major disadvantage that they activate cell cycle checkpoint mechanisms and that they are rather toxic, thus killing an important fraction of the cells. Similarly, contact inhibition (which leads to crowding of cultures) and serum withdrawal, which both lead to G1 phase arrest, perturb the experimental system by altering the susceptibility of cells to cell death induction [17].

To circumvent these problems, we sought to develop a non-toxic method for purifying cells in defined phases of the cell cycle, using an asynchronous population of cells that are in the exponential phase of growth. We generated stable transfectants that express histone H2B-GFP fusion proteins as a stoichiometric probe of chromatin (and hence DNA) content, and thus could use healthy, unmanipulated cells for fluorescence-activated cell sorter (FACS) purification of cells situated in the G1, S and G2 phases from the same culture. This novel approach for the enrichment of non-synchronized G1, S and G2 populations is suitable for the study of the impact of cell cycle on apoptosis regulation.

2. Materials and methods

2.1. Cell lines, culture and metaphase preparation

The wild-type RKO cell line and its derivative clones were cultured in Mac Coy medium supplemented with 10% fetal calf serum. To generate tetraploid and diploid clones, the cell line RKO containing 2–5% tetraploid cells was subcloned by limiting dilution (<0.01 cells per well) into diploid and tetraploid clones. One diploid clone was transfected using lipofectamine (Invitrogene) with a cDNA encoding H2B-GFP (Pharmingen) and selected in blasticidine (20 μg/ml, Invitrogene), according to the manufacturer's recommendations. Cells were then FACS-separated into subsets of cells enriched in a diploid or tetraploid DNA content to generate diploid and tetraploid H2B-GFP-expressing clones (see below for details). For karyograms, exponentially growing cells were treated with nocodazole (10 mM) for 2–4 h, harvested, and centrifuged at 1200 rpm for 5 min. The pellet was resuspended in KCl (5 mM) containing 10%FCS and incubated at 37 °C for 10 min. After 5 min centrifugation (1200 rpm), the pellet was resuspended in ethanol (75%)–acetic acid (25%) and then spread on slides, as previously described [18].

A colorimetric assay for quantification of cell viability based on the cleavage of the tetrazolium salt WST-1 (Roche Diagnostics, Germany) was used to measure the IC_{50} for cytotoxic agents. Each experiment has been performed at least three times.

2.2. Immunofluorescence and flow cytometry

Cells were allowed to adhere on poly L-Lysine microscope slides (Kindler) and fixed in 4% paraformaldehyde at room temperature for 45 min [12]. Cells were then permeabilized either with 0.05% Triton X-100 (Boehringer Mannheim) or 0.1% SDS for 10 min, washed in PBS, incubated with PBS–FCS 10% for 20 min, again washed in PBS and incubated with antibodies specific for cyclin B1 (Transduction Laboratories), activated caspase-3 (Asp175; Cell Signaling Technology), MMP-2 ((S/T)-P); Upstate, Cell Signaling Solutions), phospho-histone H3 (Ser10; Upstate, Cell Signaling Solutions), and revealed either with a goat anti-rabbit or a goat anti-mouse IgG coupled with Alexa 568 (red) or Alexa 488 (green) fluorochromes (Molecular Probes). The DNA of the cells was counterstained with Hoechst 33342 (Molecular Probes). Slides were examined with an LSM 510 microscope (Zeiss) at 63-fold magnification.

For cell cycle analyses, cells were labeled with Hoechst 33342 (2 μM, 30 min at 37 °C, Molecular Probes), which measures DNA content. At least 10,000 cells were analyzed in the cytofluorometric experiments, performed on a FACS Vantage (Becton Dickinson) equipped with a 70 μm nozzle and CellQuest software. Each experiment was done at least three times.

FACS-purification of cells with defined chromatin content was based on their H2B-GFP-dependent fluorescence (FL1, DNA content) and forward scatter characteristics (FSC, size). Roughly, cells in defined windows of FL1 and FSC (R2 and R4 for diploid cells and R3 and R5 for tetraploid cells in Fig. 2) were gated on to enrich diploid or tetraploid cells. Following the same principle, cells sorted in G1, S and G2 were gated on by defining windows in FL1 and FSC (R2 and R5 for G1, R3 and R6 for S and R4 and R7 for G2 in Fig. 3).

2.3. BrdU labeling

Cells were incubated with 30 μM of 5-bromo-2-deoxyuridine (BrdU, Sigma) for 15 min. After the pulse, the cells were washed out and resuspended in fresh medium. Cells were harvested at each time point, centrifuged at 1200 rpm for 5 min, washed in PBS and resuspended in 75% ethanol by vortexing before storage at 4 °C for 24 h. After a pepsin treatment cells were resuspended in 2 M hydrochloric acid,

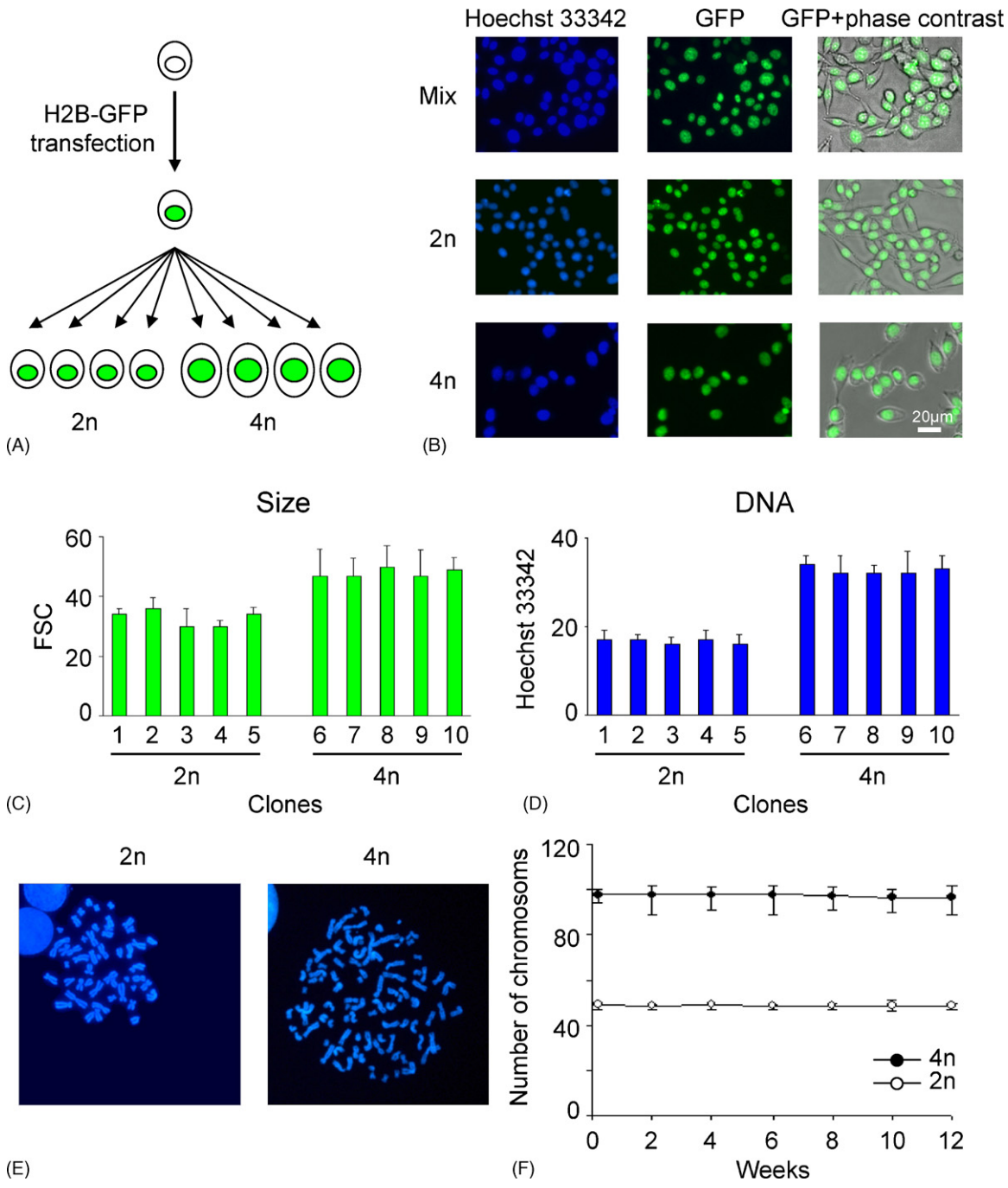

Fig. 1 – Generation of a panel of diploid and tetraploid clones stably expressing a fusion protein of histone H2B and GFP. (A and B) Strategy for the obtention of diploid and tetraploid cells. RKO cells were stably transfected with H2B-GFP and then subcloned into diploid (2*n*) and tetraploid (4*n*) cell lines, based on the fact that a small percentage of parental RKO cells (positive or negative for H2B-GFP) spontaneously become tetraploid upon prolonged in vitro culture. The morphological aspect of the cells is shown in (B), revealing that the GFP fluorescence is spatially restricted to chromatin (stained with Hoechst 33342 in blue) and that diploid and tetraploid cells differ in nuclear and cellular size. (C and D) Cell size and DNA content in 5 diploid (2*n*) and 5 tetraploid (4*n*) RKO clones. These parameters were determined by cytofluorometry, either by measuring the forward scatter (FSC in C) or the Hoechst 33342 fluorescence (D). Results are means of three experiments ± S.D. (E and F) Stability of tetraploid RKO cells. Representative karyograms of metaphase spreads stained with DAPI are shown in (E), and the evolution of chromosome counts were determined for clones 3 and 8 (C and D), revealing a stable chromosome content over prolonged culture. Values are arithmetic means ± variance ($n = 150$).

washed and incubated for 30 min with a rat anti-BrdU antibody (Oxford Biotechnology) and revealed with a goat anti-rat IgG coupled with FITC fluorochrome (Southern Biotech). Note that the fixation procedure destroyed the GFP-dependent fluorescence, meaning that the H2B-GFP expression did not interfere with the detection of BrdU incorporation. Cells were stained with propidium iodide (PI, 25 μg/ml, Sigma) and cytofluorometric analyses were performed on a FACS Vantage (Becton Dickinson). Each experiment was repeated at least three times.

3. Results

3.1. FACS purification of cells with defined chromatin content

We reasoned that it might be useful to purify cells containing a defined DNA or chromatin content from a heterogeneous population in the exponential phase of growth. Unfortunately, most, if not all fluorescent dyes like Hoechst 33342 that bind to DNA or chromatin are genotoxic and apoptogenic [19,20], and

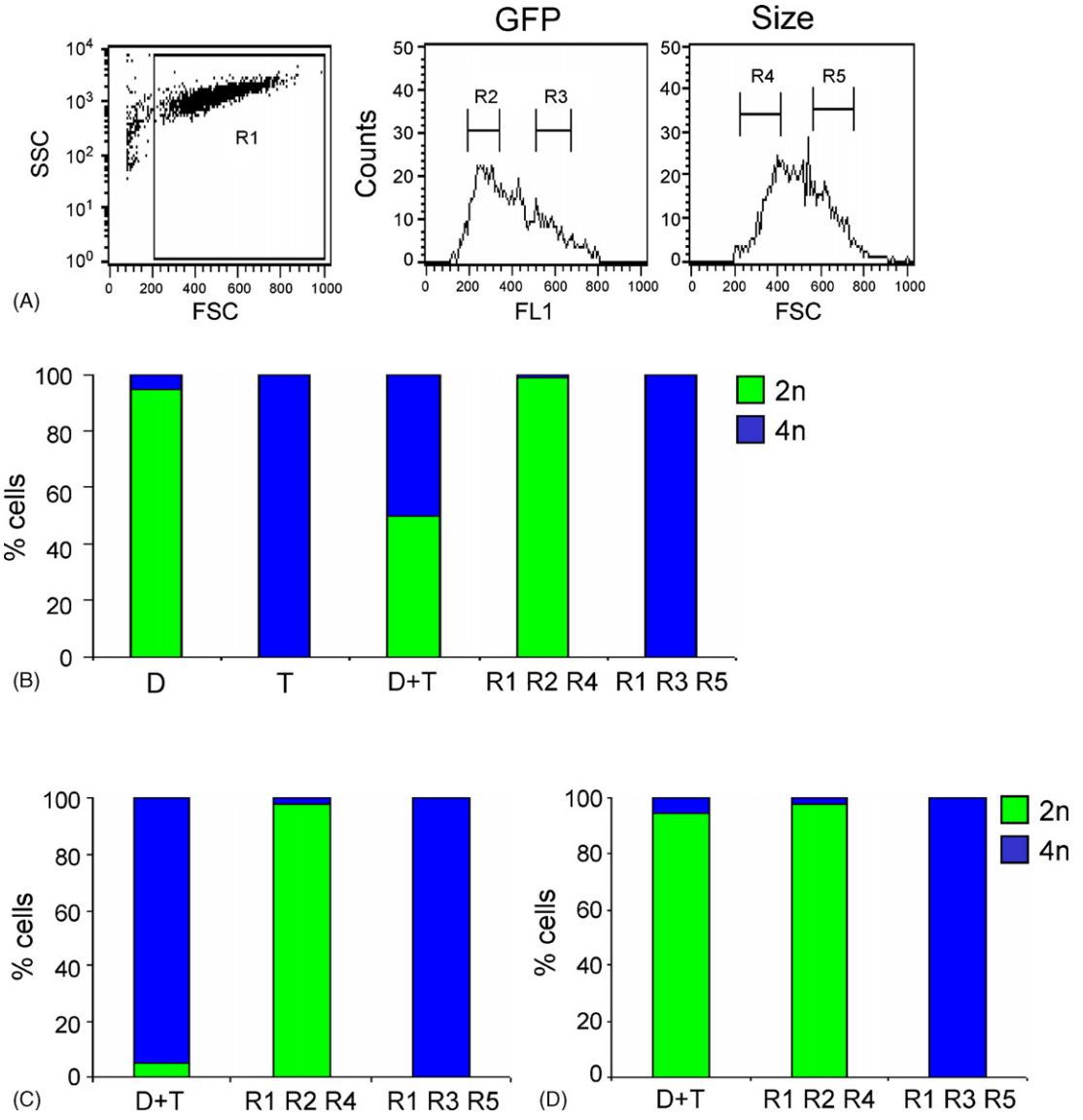

Fig. 2 – Cytofluorometric separation of diploid and tetraploid cells expressing H2B-GFP. (A and B) Separation of a 50:50 mixture of diploid (D) and tetraploid (T) cells in the FACS. Two representative RKO clones were admixed at a 1:1 ratio and subjected to FACS analysis gating on the live cell population (R1 in the plot of the side-scatter, SSC, vs. forward scatter, FSC) on cells of defined chromosome content (R2 and R3 in the histogram describing the frequency distribution of the GFP fluorescence) and cells of separable size (R4 and R5 in the histogram of FSC) (see Section 2 for details). The portion of diploid and tetraploid cells was determined by chromosome counting (as in Fig. 1E and F) after mixing (D + T), before and after FACS purification. Note that the cells that are found in gates R1, R2 and R4 are >98% diploid, while those found in gates R1, R3 and R5 are never diploid. (C and D) Separation of 19:1 (C) or 1:19 (D) mixtures of diploid and tetraploid cells in the FACS. Cells were admixed at the indicated ratios, followed by FACS purification as in (A), and a satisfactory degree of purity was obtained for diploid and tetraploid cells.

thus are not suitable for the recovery of live cells representative of the normal state. Therefore, we decided to generate cells containing a non-toxic histone-H2B-GFP fusion protein that partitions into chromatin and allows for the in vivo study of chromosome movement [21]. We took advantage of a diploid human cell line (RKO) that spontaneously exhibits a low rate of tetraploidization (~0.2% per generation), meaning that ~2–5% of the cells are tetraploid at a given time point [22]. We subcloned the H2B-GFP-expressing parental cell line into diploid and tetraploid clones (Fig. 1A), determined that these clones effectively retained the H2B-GFP fluorescence (Fig. 1B), and differed in their size (Fig. 1C), DNA (Fig. 1D) and chromosome content (Fig. 1E), which remained stable upon prolonged culture (Fig. 1F). Then, we admixed diploid and tetraploid cells at different ratios (1:1, 19:1, 1:19) and used the fluorescence-activated cell sorter to purify the populations, based on their forward scatter characteristics (FSC) and H2B-GFP-dependent fluorescence (Fig. 2A). This allowed us to separate cell populations with a diploid or tetraploid genome (as determined by chromosome counting (Fig. 2), illustrating

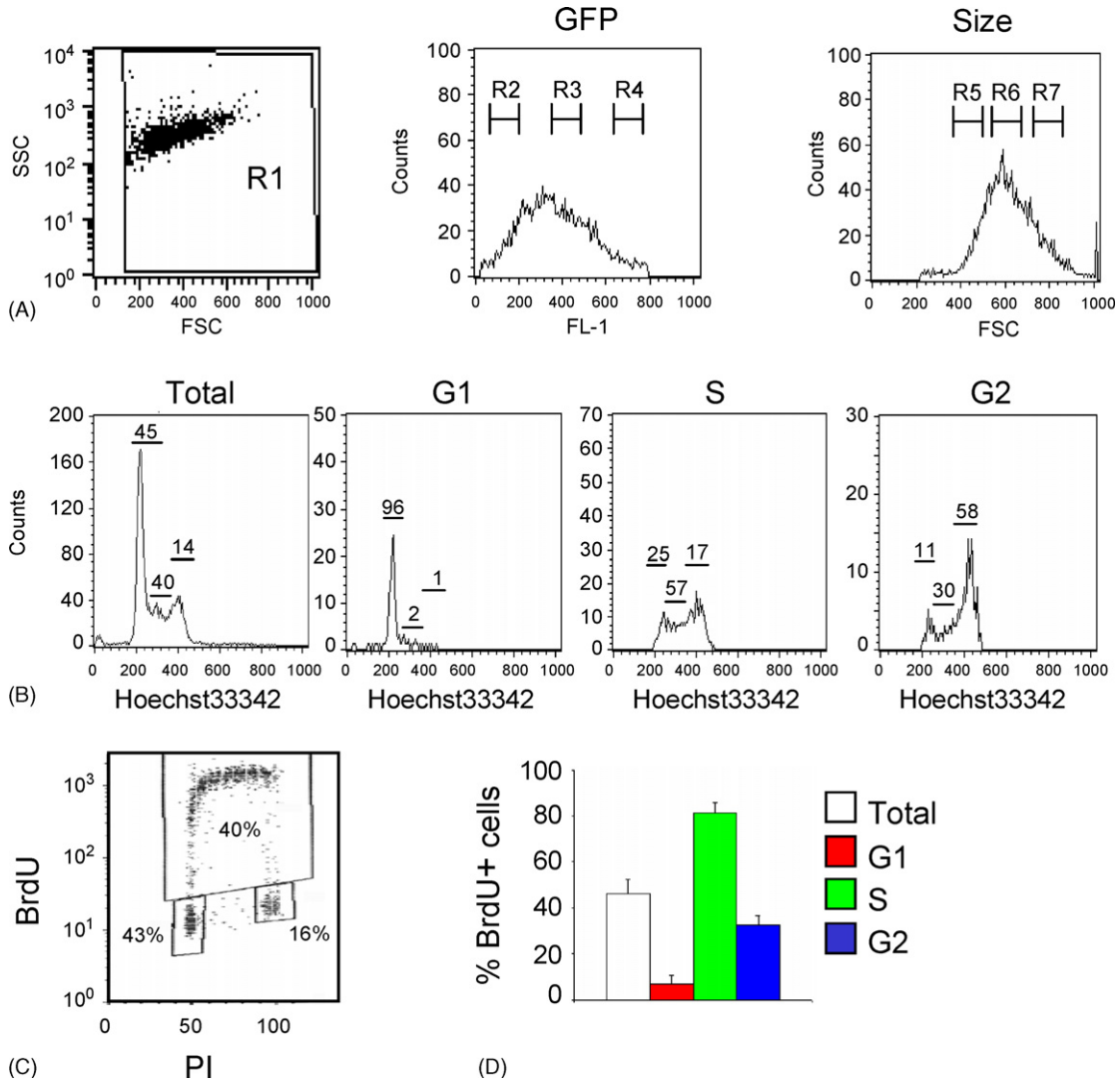

Fig. 3 – Cytofluorometric separation of diploid cells expressing H2B-GFP in different phase of the cell cycle. (A) Method of separation. Diploid RKO cells transfected with H2B-GFP (clone 3 in Fig. 1) in the logarithmic growth phase were subjected to FACS analysis, while gating on normal-sized cells (R1 in the FSC vs. SSC plot), as well as three categories of cells differing in their GFP fluorescence (R2, R3, R4) and FSC (R5, R6, R7). The cells were then sorted into three categories, namely bona fide G1 (R1 + R2 + R5), S (R1 + R3 + R6) and G2 (R1 + R4 + R7) (see Section 2 for details). Two alternative methods were used to determine the purity of the cells purified as in (A). As a first possibility (B), the cells were immediately labeled with Hoechst 33342 and then subjected to cytofluorometric determination of DNA content. Numbers on each gate indicate the percentage of cells in the G1, S and G2/M phases of the cell cycle. As a second method of quality control (C and D), the cells were pulsed for 15 min with BrdU, then purified in the cytofluorometer, fixed and permeabilized using a protocol that destroys the GFP-dependent fluorescence. Then, the cells were stained to measure DNA content (with propidium iodide, PI) and BrdU incorporation (with a suitable BrdU monoclonal antibody revealed by a secondary FITC-labeled conjugate). The Hoechst 33342 and PI staining profiles were very similar (B and C) and the percentages of cells capable of incorporating BrdU are depicted in (D).

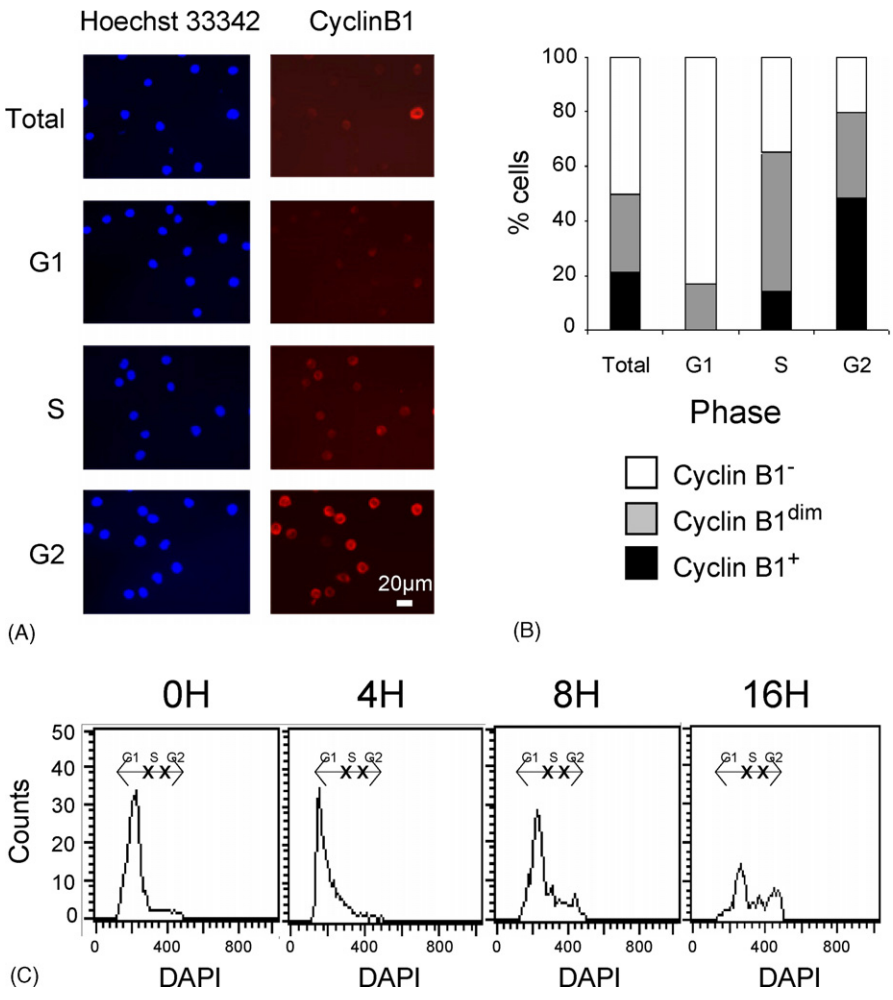

Fig. 4 – FACS-purified cells are viable and resume cell cycle progressing. Cells purified according to their position in the cell cycle (as in Fig. 3) were stained for the detection of intracellular cyclin B1 (A), and the frequency of cells exhibiting a weak or intense cyclin B1 staining was determined by two independent investigators (B). Cell cycle progression after FACS purification was also assessed (C). Cells showing a similar degree of enrichment in the G1 phase of the cell cycle as in Fig. 3 were subjected to culture during the indicated interval, followed by cytofluorometric determination of the DNA content after DAPI staining.

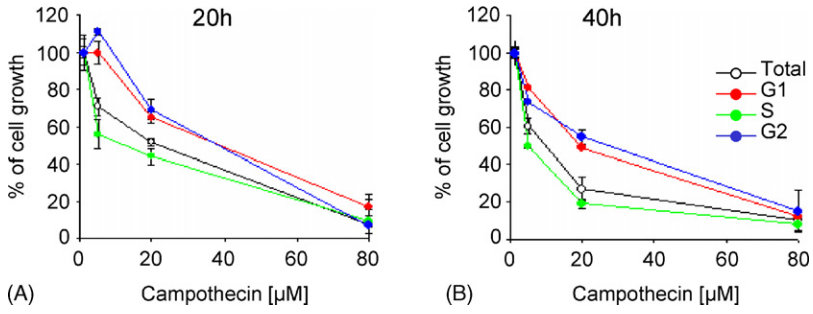

Fig. 5 – Cell cycle dependent effects of camptothecin. Diploid H2B-GFP-expressing cells subjected to purification of distinct phases of the cell cycle as in Fig. 3 were treated with the indicated doses of camptothecin, either for 20 h (A) or for 40 h (B), followed by determination of cell survival with a modified tetrazolium assay (see Section 2). Data are means ± S.D. of triplicates. This experiment has been performed three times, and similar results were obtained.

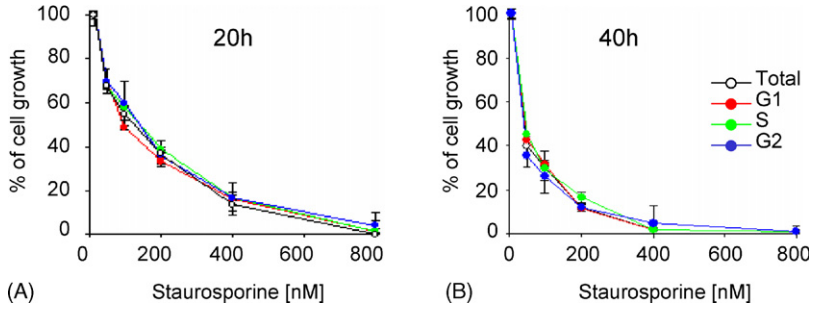

Fig. 6 – Lack of cell cycle dependent effects of staurosporine. Cells were purified as in Fig. 3 and then treated for 20 h (A) or 40 h (B) with staurosporine, followed by the measurement of viability with a modified tetrazolium assay as in Fig. 5. Results are means of three experiments ± S.D.

the possibility of FACS-purifying cells based on their chromatin content.

3.2. *FACS purification of non-synchronized cells in distinct phases of the cell cycle*

In a subsequent series of experiments, we concentrated on one representative diploid RKO clone expressing H2B-GFP and used again the FSC and H2B-GFP fluorescence to separate the cells as a function of the cell cycle (G1, S, G2) (Fig. 3A). This procedure led to the enrichment of reasonably homogenous populations, as determined by two independent methods of DNA quantification, namely with Hoechst 33342 (Fig. 3B) or with propidium iodide plus BrdU staining (Fig. 3C). For the Hoechst 33342 method, the purity was >95, ~60 and ~60% for G1, S and G2, respectively. For the BrdU method, the purity was >95, ~80 and ~70% for G1, S and G2, respectively. In addition, it was mostly the G2 population that, as to be expected, expressed cyclin B1 (which is absent in G1) (Fig. 4A and B). The FACS-purified G2 population was devoid of cells in the M phase, as indicated by negative staining (<1%) with mitotic markers such as MMP2 or phosphorylated histone H3 (H3P) (not shown), in line with the fact that the starting material was composed only by adherent cells (and hence did not include M phases).

Importantly, the cells enriched by this method in G1, S or G2 were fully viable (>95% in each phase of the cell cycle) as indicated by a normal mitochondrial transmembrane potential (not shown) and were highly efficient in resuming the cell cycle upon reculture (Fig. 4C), indicating that this unique method of purification did not perturb the basic metabolic and proliferative functions.

3.3. *Cell cycle differences in apoptosis susceptibility*

Once a satisfactory experimental system of cell cycle purification had been established, we tested the susceptibility of G1, S or G2 populations to a panel of cytotoxic drugs. Upon, reculture, less than 2% of the purified populations spontaneously died from apoptosis. However, the cells were readily susceptible to the death-inducing and growth-inhibitory effects of cytotoxic drugs. Cells in the S phase are particularly sensitive to growth inhibition by the topoisomerase type I inhibitor camptothecin (Fig. 5A and B). In strict contrast, there was no difference whatsoever among the distinct phases of the cell cycle when the effect of the general tyrosine kinase inhibitor staurosporine was assessed (Fig. 6A and B). These results could be confirmed when apoptosis was assessed by measuring the frequency of cells staining positively for activated caspase-3 after treatment with camptothecin or staurosporine. While there was no difference in caspase-3 activation by staurosporine in the distinct phases of the cell cycle, there was a marked increase in caspase-3 activation in the S phase (as compared to the G1 or G2 phases), when camptothecin was the apoptosis inducer (Fig. 7). Having validated the utility of the procedure for the induction of cell cycle-dependent apoptosis, we tested whether the IKK inhibitor BAY11-7082 would induce apoptosis in a cell cycle-specific fashion. Unexpectedly, it appeared that cells in the G2

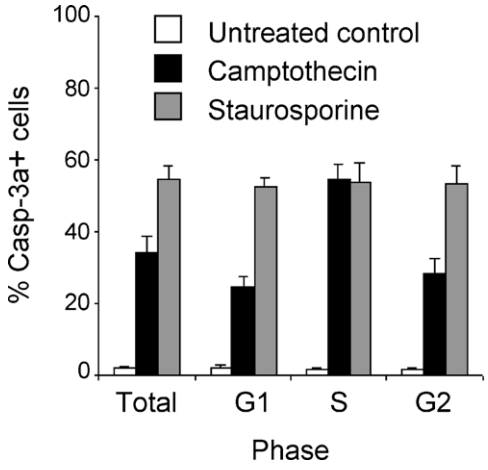

Fig. 7 – Cell cycle-dependent caspase activation. Cells purified as in Fig. 3, were cultured overnight in the absence or presence of the indicated agents (as in Figs. 5 and 6) and the cells were subjected to immunofluorescence stainings for the detection of activated caspase-3 (Casp-3a). The percentage of cells positive for Casp-3a was determined for each of the fractions cultured in the absence of apoptosis inducers (control) or in the presence of camptothecin (40 μM) or staurosporine (150 nM). Results are means of three experiments ± S.D.

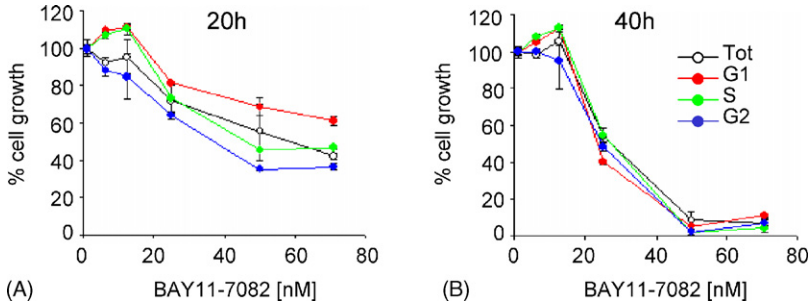

Fig. 8 – Cell cycle-dependent effect of BAY11-7082. Cells purified as in Fig. 3 were cultured overnight in the absence or presence of the indicated concentration of BAY11-7082 and the degree of survival was determined (as in Figs. 5 and 6). Results are means of three experiments ± S.D.

phase were more susceptible to BAY11-7082 ($IC_{50} \sim 40$ nM) than cells in G1 ($IC_{50} > 80$ nM), while S phase cells and unseparated control cells comprising all phases of the cell cycle exhibited an intermediate behavior. This strong effect was observed in short-term (20 h) experiments (Fig. 8A), whereas the cell cycle specificity disappears upon long-term (40 h) culture (Fig. 8B).

4. Discussion

The data contained in this paper delineate a novel procedure for the purification of cells in the G1, S or G2 phases of the cell cycle (Figs. 1–4). At difference with commonly used methods, this procedure does not involve any pharmacological manipulation (cell cycle arrest), is non-toxic and does not perturb the general apoptosis susceptibility of the cells and is probably applicable to all cell lines able that support the stable integration of the H2B-GFP-encoding vector. Although the method requires an initial investment, namely the generation of stably H2B-GFP-expressing clones, it yields cell cycle-specific fractions with an acceptable degree of purity (>95% for G1, 80% for S and ~70% for G2) (Figs. 3 and 4). The cells that have been purified show less than 2% mortality, even after reculture (Fig. 7) and readily resume the cell cycle (Fig. 4C). Using this method, we evaluated the growth-inhibitory effects of a selected panel of cytotoxic drugs. As to be expected, staurosporine had no cell cycle specificity and equally suppressed the growth of G1, S and G2 cells (Fig. 6) and induced a similar degree of caspase-3 activation (Fig. 7). In contrast, camptothecin preferentially inhibited the growth in the S phase (Fig. 5A and B) and induced the apoptotic death more efficiently in the S phase than in G1 or G2 (Fig. 7), in line with previous studies [23,24]. We also shown for the first time that the IKK inhibitor BAY11-7082 is more cytotoxic for cells in the G2 phase of the cell cycle (Fig. 8), suggesting that NF-κB (whose activation depends on IKK) is particular critical for the survival of cells in this phase of the cell division cycle.

In conclusion, we developed a novel, experimentally exploitable procedure for the enrichment of cells in distinct phase of the cell cycle. This method may be particularly useful for studying the intricate interplay between apoptosis and cell cycle regulation.

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