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p53 facilitates pRb cleavage in IL-3-deprived cells: novel pro-apoptotic activity of p53

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In the interleukin-3 (IL-3)-dependent lymphoid cell line DA-1, functional p53 is required for efficient apoptosis in response to IL-3 withdrawal. Activation of p53 in these cells, by either DNA damage or p53 overexpression, results in a vital growth arrest in the presence of IL-3 and in accelerated apoptosis in its absence. Thus, IL-3 can control the choice between p53-dependent cell-cycle arrest and apoptosis. Here we report that the cross-talk between p53 and IL-3 involves joint control of pRb cleavage and degradation. Depletion of IL-3 results in caspase-mediated pRb cleavage, occurring preferentially within cells which express functional p53. Moreover, pRb can be cleaved efficiently by extracts prepared from DA-1 cells but not from their derivatives which lack p53 function. Inactivation of pRb through expression of the human papillomavirus (HPV) E7 oncogene overrides the effect of IL-3 in a p53-dependent manner. Our data suggest a novel role for p53 in the regulation of cell death and a novel mechanism for the cooperation between p53 and survival factor deprivation. Thus, p53 makes cells permissive to pRb cleavage, probably by controlling the potential activity of a pRb-cleaving caspase, whereas IL-3 withdrawal provides signals that turn on this potential activity and lead to the actual cleavage and subsequent degradation of pRb. Elimination of a presumptive anti-apoptotic effect of pRb may then facilitate conversion of p53-mediated growth arrest into apoptosis.

Keywords: apoptosis/caspase/interleukin-3/p53/retinoblastoma

Introduction

The p53 tumor suppressor gene undergoes frequent mutational inactivation in human tumors (for recent reviews on p53 see Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). The tumor-associated mutations most probably serve to inactivate the tumor-suppressor functions of the wild-type (wt) p53 protein. Biochemically, p53 is a potent transcription factor, capable of sequence-specific transactivation (SST) of target genes containing distinct p53 recognition motifs.

In normal cells, the p53 protein is presumably latent. Furthermore, its steady-state concentration is extremely low, owing to a very short half-life. A variety of stress signals, including genotoxic stress, cause p53 stabilization and consequent accumulation in a biochemically activated form. Activation of p53 in response to DNA damage most probably serves to prevent the propagation of cells bearing potentially dangerous genetic lesions (Lane, 1992). In certain situations p53 activation results in a transient growth arrest, presumably allowing the damaged DNA to be properly repaired before the cell re-enters the cell cycle and replicates its genome. In other instances, activation of p53 can permanently remove damaged cells from the replicative pool, either through induction of a long-lasting G_1 arrest or by the stimulation of apoptosis.

The p53 protein is an important regulator of apoptosis under a variety of physiological and pathological conditions (reviewed in Bates and Vousden, 1996; Gottlieb and Oren, 1996; Canman and Kastan, 1997; Wyllie, 1997). At least some of the pro-apoptotic effects of p53 are mediated through its SST function (Sabbatini *et al*., 1995; Yonish-Rouach *et al*., 1995; Attardi *et al*., 1996; Polyak *et al*., 1997). However, other less well-defined activities of p53 also play a role in p53-mediated apoptosis (Caelles *et al*., 1994; Shen and Shenk, 1994; Wagner *et al*., 1994; Haupt *et al*., 1995a; Ishioka *et al*., 1995; Gottlieb *et al*., 1996).

We have reported previously a cross-talk between p53 and interleukin-3 (IL-3) in control of the fate of DA-1 mouse lymphoma cells. DA-1 cells undergo rapid apoptosis in the absence of IL-3, but this process is delayed significantly when their endogenous wt p53 is inactivated prior to IL-3 withdrawal (Gottlieb *et al*., 1994). These findings, along with work by others (Lotem and Sachs, 1993; Zhu *et al*., 1994; Blandino *et al*., 1995), suggested that p53 can modulate the cellular response to IL-3 withdrawal. Conversely, IL-3 can regulate the cellular outcome of p53 activation. Thus, exposure of DA-1 cells to moderate DNA damage causes a prominent accumulation of transcriptionally active p53; in the presence of IL-3, this leads to a transient viable growth arrest, whereas in its absence the cells undergo very rapid apoptosis (Gottlieb *et al*., 1996). In line with similar observations by Canman *et al*. (1995), this implies that IL-3 can dictate whether activation of p53 in hematopoietic cells results in death or only in a viable arrest. Interestingly, in DA-1 cells exposed to lethal doses of ionizing radiation (IR), the onset of apoptosis is preceded by re-entry of G_1-arrested cells into the cell cycle (Gottlieb *et al.*, 1996). Thus there appears to exist an inverse relationship between stable cell-cycle arrest and p53-mediated apoptosis (Qin *et al*., 1994; Shan and Lee, 1994; Wu and Levine, 1994; Canman *et al*., 1995; Haupt *et al*., 1995b; Gottlieb *et al*., 1996).

The retinoblastoma gene product (pRb) is a phosphoprotein which can both regulate cell-cycle progression and inhibit apoptosis (reviewed in Herwig and Strauss, 1997; Wang, 1997). pRb can be regulated through phosphorylation by cyclin-dependent kinases (CDKs). When hyper-

phosphorylated, pRb loses its ability to block cell-cycle progression. Upon dephosphorylation, pRb is activated and induces growth arrest at the G_1 phase of the cycle. Interestingly, pRb activity can be regulated by p53 through the induction of $p21^{Waf1}$, which is a p53 target gene and a CDK inhibitor. Increased levels of $p21^{Waf1}$ result in an active, underphosphorylated pRb that can mediate G_1 arrest. Overexpression of $p21^{Waf1}$ can inhibit apoptosis (Poluha *et al*., 1996; Wang and Walsh, 1996; Gorospe *et al*., 1997), presumably through blocking pRb phosphorylation. In addition, inactivation of $p21^{Waf1}$ in colorectal cancer cells changes their response to p53 overexpression from growth arrest to apoptosis (Polyak *et al*., 1996). Hence, the functional status of pRb may be an important determinant in dictating the cellular outcome of p53 activation.

We wished to determine whether pRb is also involved in the cross-talk between p53 and IL-3 in the control of apoptosis. To that end, pRb status was monitored in DA-1 cells following different treatments that result in either apoptosis or growth arrest. We report that pRb is subject to caspase-mediated proteolytic cleavage in cells undergoing apoptosis in response to IL-3 withdrawal. Efficient intracellular pRb cleavage requires the presence of functional wt p53, and is accelerated under conditions that cause p53 activation. Remarkably, exogenous pRb can be cleaved efficiently by cell-free extracts from DA-1 cells possessing functional wt p53, but not from cells in which p53 activity has been compromised. These findings suggest a novel role for p53 in the regulation of cell death and a molecular mechanism for cooperation between p53 and IL-3 deprivation. The presence of functional p53 makes the cells permissive to pRb cleavage, probably by maintaining a pRb-cleaving caspase in a potentially activatable state. In turn, IL-3 withdrawal provides the signals that actually unleash this caspase activity and lead to the cleavage and subsequent degradation of pRb. In further support of the role of IL-3 as a protector of pRb integrity, abrogation of pRb function through expression of the human papillomavirus (HPV) E7 oncogene overrides the protective effect of IL-3 in DA-1 cells. Interestingly, cells lacking functional p53 are immune to the adverse effect of HPV E7. pRb is a key mediator of p53-dependent growth arrest and at the same time a presumptive inhibitor of apoptosis. Inactivation of pRb, as occurs in response to IL-3 withdrawal, may thus facilitate conversion of p53-mediated growth arrest into apoptosis.

Results

Modulation of pRb cleavage by IL-3

DA-1 is an IL-3-dependent lymphoid cell line (Ihle, 1984). DA-1 cells undergo apoptosis upon IL-3 withdrawal, and the efficiency of this process depends significantly on the presence of functional p53 (Gottlieb *et al*., 1994). DA-1 cells can undergo p53-dependent apoptosis even in the presence of IL-3, when exposed to high doses (>3 Gy) of IR (Gottlieb *et al*., 1996). Exposure of DA-1 cells to lower doses of IR (e.g. 1 Gy) results in the accumulation of p53 and transactivation of its target genes, similar to that observed with apoptosis-inducing doses of IR; however, as long as IL-3 is present in the medium these low doses elicit only a transient G_1 arrest, and very little apoptosis is evident (Gottlieb *et al*., 1996). Hence, similar to what has been observed in another hematopoietic cell line, Baf-3 (Collins *et al*., 1992; Canman *et al*., 1995), IL-3 can protect against p53-mediated apoptosis.

Withdrawal of IL-3 from Baf-3 cells results in decreased accumulation of $p21^{Waf1}$, an inhibitor of pRb phosphorylation (Canman *et al*., 1995). pRb can confer protection against p53-dependent and p53-independent apoptosis (Qin *et al*., 1994; Haas-Kogan *et al*., 1995; Haupt *et al*., 1995b; reviewed in Herwig and Strauss, 1997; Wang, 1997). We therefore wished to investigate possible changes in pRb status following IL-3 withdrawal.

The first set of experiments was performed using DIV6 cells. DIV6 were derived from DA-1 cells by retroviral transduction with a temperature-sensitive (ts) p53 mutant, p53val135 (Gottlieb *et al*., 1994, 1996). When these cells are maintained at the permissive temperature of 32°C, the SST activity of the ts p53 is strongly stimulated. On the one hand, in the presence of IL-3, this high activity of p53 induces only a vital growth arrest without any signs of apoptosis (Gottlieb *et al*., 1996). On the other hand, in the absence of IL-3 DIV6 cells undergo apoptosis at 32°C at a significantly faster rate than parental DA-1 cells.

DIV6 cells were incubated at the permissive (32°C) or non-permissive (37°C) temperature in the presence or absence of IL-3. Total cell extracts were prepared and subjected to SDS–PAGE followed by Western blot analysis with anti-pRb antibodies (Figure 1A). At 37°C, when the ts p53 behaves largely like a typical mutant p53, pRb was found to be mostly hyperphosphorylated (Figure 1A, lane 1, form a). Conversely, in cells maintained at 32°C in the presence of IL-3, pRb was mostly underphosphorylated (Figure 1A, lane 3, form b; Figure 1B, lanes 3, 5 and 6). This is probably due to p53-mediated *waf1* induction, and is consistent with the fact that these cells undergo growth arrest at 32°C (Gottlieb *et al*., 1996). Interestingly, in DIV6 cells undergoing accelerated apoptosis at 32°C upon IL-3 deprivation, a new form of pRb became apparent; this form (Figure 1A, lane 4, form c) migrated faster than full-length underphosphorylated pRb. The position of this band suggests that it represents a proteolytic cleavage product of pRb. Indeed, it has recently been reported that pRb can be cleaved by a caspase during apoptosis, resulting in the appearance of a faster migrating band and subsequently also of smaller proteolytic products (Janicke *et al*., 1996; Tan *et al*., 1997; Tan and Wang, 1998). In line with these findings, when DIV6 cells are incubated at 32°C without IL-3 for a longer time, smaller forms of pRb can also be observed (data not shown), concomitant with a reduction in the overall amount of detectable pRb (Figure 1B, lane 4). Form c of pRb is also detectable in IL-3-deprived DIV6 cells at 37°C, albeit in significantly lower quantities (Figure 1A and B, lane 2).

To confirm that the faster migrating form was indeed due to caspase-mediated cleavage of pRb, DIV6 cells were incubated at 32°C with or without IL-3 in the presence of the modified tetrapeptide DEVD-fmk, a potent caspase inhibitor. The appearance of the faster migrating form of pRb in the absence of IL-3, as well as its further degradation, were efficiently blocked by DEVD-fmk (Figure 1B, compare lane 6 with lane 4). Not surprisingly, the apoptotic process was also significantly attenuated.

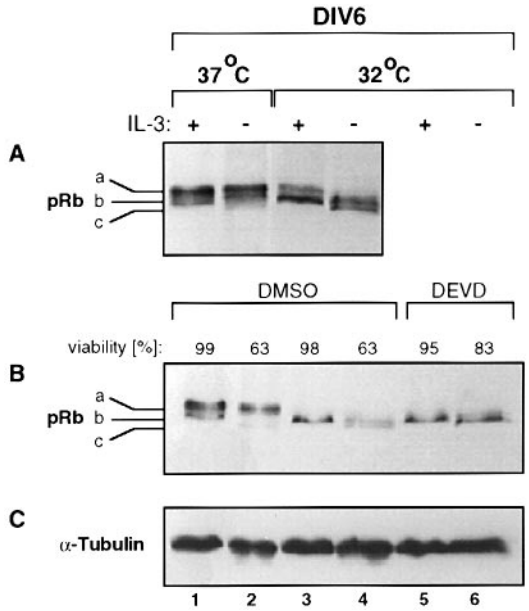

Fig. 1. Analysis of changes in pRb in DIV6 cells. DIV6 are derivatives of DA-1 cells, containing the ts mutant p53val135. (**A**) Cells were maintained for 12 h at the indicated temperature, in the presence or absence of IL-3. Cell extracts were subjected to Western blot analysis with pRb-specific antibodies. The positions of hyperphosphorylated pRb (a), underphosphorylated pRb (b) and the faster migrating form of pRb (c) are indicated. (**B**) Cells were maintained for 18 h at either 37°C or 32°C, with or without IL-3, as indicated at the top of the figure. A caspase inhibitor (DEVD-fmk, 200 μM final concentration) was included in the indicated 32°C cultures (lanes 5 and 6). To the other cultures, DMSO was added as a control for non-specific solvent effects. Prior to extraction, viability was analyzed microscopically by trypan blue exclusion. It is of note that the death of parental DA-1 cells in the absence of IL-3 is much faster at 37°C than at 32°C (Gottlieb *et al.*, 1994). Hence, the extent of cell death in the DIV6 culture corresponding to lane 4 is actually augmented markedly relative to DA-1 cells maintained under identical conditions. (**C**) The same blot as in panel B was reprobed with anti-α-tubulin antibodies as a control for equal loading.

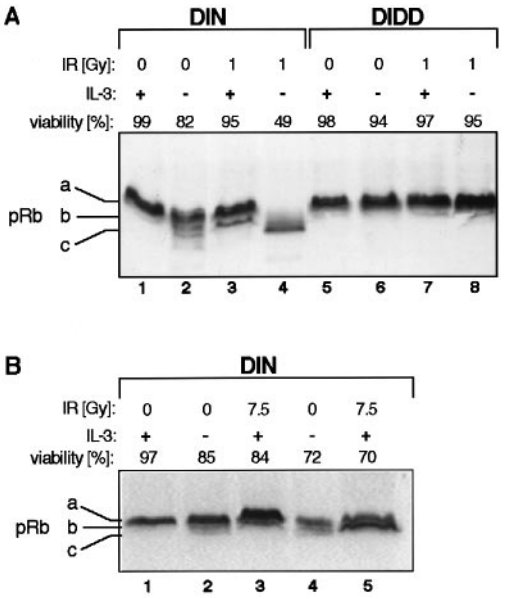

Fig. 2. Western blot analysis of pRb in DA-1 cells containing the gene for neomycin resistance alone (DIN) or together with a dominant negative C-terminal fragment of p53 (DIDD). (**A**) Cells were washed and resuspended in the presence or absence of IL-3 and 6 h later were exposed to the indicated amount of IR (γ radiation from a ^{60}Co source). Viability was analyzed microscopically by trypan blue exclusion 3 h after treatment. (**B**) DIN cells were either maintained in medium without IL-3 or exposed to 7.5 Gy of IR in medium containing IL-3. Samples in panel B were harvested for analysis at the following time points after treatment: lanes 1–3, 10 h; lane 4, 13 h; lane 5, 24 h. In each case (A and B) total protein extracts for Western blot analysis were prepared immediately after the viability count of each sample. The different forms of pRb are as in Figure 1.

Hence, IL-3 deprivation results in caspase-mediated pRb cleavage in DA-1 cells.

p53 activity enables efficient pRb cleavage

DIN cells (Gottlieb *et al*., 1994, 1996) were derived through infection of DA-1 with a virus encoding neomycin resistance only, and are thus essentially equivalent to parental DA-1. Changes in pRb in these cells under different conditions were followed by Western blot analysis. IL-3 withdrawal from DIN cells results in the appearance of the cleaved form of pRb (Figure 2A, form c, lane 2) as early as 9 h after IL-3 withdrawal, when the vast majority of the cells are still viable. A smaller form of pRb, possibly a result of further proteolytic cleavage, is also observed.

Exposure of DIN cells to 1 Gy of IR in the presence of IL-3 causes an increase in p53 protein levels, resulting in a transient growth arrest but no significant apoptosis (Gottlieb *et al*., 1996). However, in the absence of IL-3 the same amount of IR results in very rapid cell death (Figure 2A, lane 4), the entire population being dead within 6 h (data not shown). This is similar to that described for Baf-3 cells (Canman *et al*., 1995). Irradiation of DIN cells with 1 Gy in the presence of IL-3 results in partial pRb dephosphorylation (form b), but no pRb cleavage (Figure 2A, lane 3). In contrast, in the absence of IL-3 there is a rapid disappearance of full-length pRb, which is cleaved and further degraded; complete cleavage is observed 3 h after irradiation, when half of the cells are still viable (Figure 2A, lane 4). Exposure of DIN cells to 1 Gy of IR entails a pronounced induction of p53 activity (Gottlieb *et al*., 1996). In line with the observations described above for DIV6 cells these data demonstrate that, upon activation of p53, pRb is cleaved rapidly in the absence of IL-3 and dephosphorylated but not cleaved in the presence of IL-3.

Next, we wished to find out whether rapid pRb cleavage is preferentially induced by IL-3 withdrawal, rather than being a secondary by-product of the apoptotic process. DIN cells undergo apoptosis even in the presence of IL-3, providing they are exposed to high doses of IR (Gottlieb *et al*., 1996). The pattern of pRb in IL-3-deprived DIN cells was therefore compared, at early stages of the apoptotic process, with that in parallel cultures exposed to 7.5 Gy of IR in the presence of IL-3. pRb cleavage was prominent in IL-3-deprived cells even when the vast majority of the culture was still viable (Figure 2B, form c, lane 2). In stark contrast, no pRb cleavage was detectable in the parallel irradiated culture, even though the rate of cell death was practically identical (Figure 2B, lane 3). As expected, the irradiated cells contained a substantial fraction of underphosphorylated pRb, presumably due to p53-dependent induction of *waf1* expression. A similar picture was revealed at a somewhat more advanced, albeit still relatively early, stage of the apoptotic process

(Figure 2B, compare lane 4 with lane 5). Note that at this point a clear reduction in the overall amount of pRb could already be discerned in the IL-3-deprived culture (Figure 2B, lane 4). These data argue strongly that IL-3 deprivation serves as a specific trigger for pRb cleavage, although it remains possible that similar cleavage may occur as a late by-product of the apoptosis elicited by other stimuli.

The DIV6 data depicted in Figure 1 indicate that pRb cleavage and the subsequent reduction in overall pRb concentration occur more efficiently in the presence of excess functional p53. There is, however, also some pRb cleavage at 37°C. This could imply that within DIV6 cells p53 is not required for cleavage and exerts only a limited accelerating effect on this IL-3-regulated process. Alternatively, the cleavage observed at 37°C might be due to residual p53 activity in DIV6 cells, where the dominant negative effect of the ts p53 appears to be incomplete (Gottlieb *et al*., 1994). To further clarify this issue, use was made of DIDD1 cells which are derivatives of DA-1 carrying a more effective inhibitor of p53 function.

DIDD1 cells were obtained through infection of DA-1 with a retrovirus encoding the C-terminal fragment of p53, a potent dominant negative inhibitor of p53 (Gottlieb *et al*., 1994); this infection was carried out in parallel with the one employed to generate DIN cells, where the retrovirus encoded only neomycin resistance. DIDD1 cells possess significantly increased resistance to apoptosis induced by IL-3 withdrawal, exposure to IR or a combination of both (Gottlieb *et al*., 1996; Figure 2A, lanes 6–8). Importantly, induction of pRb cleavage in the absence of IL-3 is also severely compromised (Figure 2A, compare lanes 2 and 6 with lanes 4 and 8). Taken together, the data imply that functional wt p53 is required for efficient pRb cleavage in response to IL-3 deprivation. Moreover, further p53 activation (e.g. in response to DNA damage) accelerates this process.

pRb is member of a family of proteins which includes p107 and p130. Neither of these two proteins undergoes cleavage in apoptotic DA-1 cells (data not shown). The primary cleavage site of pRb has been mapped to its C-terminus (Janicke *et al*., 1996; Chen *et al*., 1997); this site is not conserved in p107 and p130. It therefore seems that pRb, unlike these other two members of the family, is uniquely regulated by caspase cleavage.

Loss of pRb function promotes p53-dependent death of DA-1 cells

The findings described above suggest that loss of pRb function, as is assumed to occur when pRb is cleaved and subsequently degraded, facilitates apoptosis in DA-1 cells. Furthermore, the findings imply that maintenance of pRb integrity and functionality may be an important component of the survival effect of IL-3 in DA-1 cells. If that conjecture is correct, then abrogation of pRb function may promote apoptosis even when IL-3 is present.

The E7 oncoprotein of the high-risk HPVs can bind and inactivate pRb, as well as other members of the pRb family, and was shown in several systems to induce p53-dependent apoptosis (Howes *et al*., 1994; Pan and Griep, 1994). The E7 protein of HPV type 16 was therefore utilized in order to test whether pRb inactivation could override the protective effect of IL-3. To that end recombinant retroviruses were constructed, encoding puromycin resistance (puro) either alone (control virus) or together with wt E7 or mutant E7 (24GLYE7, cysteine to glycine substitution at position 24; Edmonds and Vousden, 1989). The 24GLYE7 mutant can not bind pRb, or its related proteins p107 and p130 (Davies *et al*., 1993 for *in vitro* analysis; Figure 4C for *in vivo* data). Virus titers were not determined on the standard NIH 3T3 cells, but rather on SVT2 cells (SV40-transformed derivatives of NIH 3T3). The SV40 large T protein, expressed in SVT2, binds and inactivates both pRb and p53; hence, these cells are expected to be immune to the detrimental effects of E7, thereby allowing a more reliable determination of E7 virus titers.

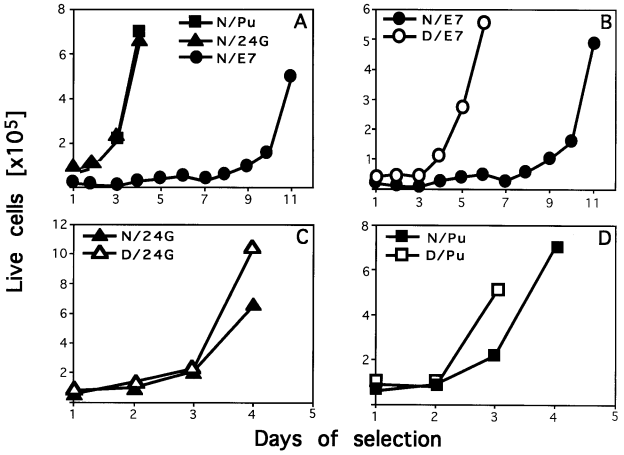

Fig. 3. Growth curves of retrovirally infected DA-1 cells during puromycin selection. DA-1 cells were infected with retroviruses expressing neomycin resistance either alone (N) or in combination with a dominant negative C-terminal fragment of p53, p53DD (D); the corresponding G418-resistant populations were designated DIN and DIDD, respectively. Following G418 selection for 5 days, 10^5 cells of DIN (N/..) or DIDD (D/..) were subjected to a second round of infection with viruses encoding puromycin resistance either alone (../Pu), or together with wt HPV type-16 E7 (../E7) or an E7 mutant, E724GLY (../24G). Infected cells were subjected to puromycin selection. Live cells were counted daily. (**A**) DIN cells reinfected with each of the different puromycin viruses. (**B**) DIN and DIDD cells infected with wt E7. (**C**) DIN and DIDD infected with E724GLY. (**D**) DIN and DIDD cells infected with control virus encoding puromycin resistance only.

A two-step experiment was performed. First, DA-1 cells were infected with control (neomycin-resistance) virus or with virus encoding the dominant negative p53 mutant DD. Fresh populations of DIN and DIDD cells, respectively, were obtained. After 5 days of G418 selection these cells were re-infected with comparable inoccula of the various puro retroviruses described above. Following infection of 10^5 cells with each virus, cells were subjected to drug selection and the total number of live cells was counted every day until viability reached >50%. Figure 3A depicts the data for DIN cells. Cells infected with either control (Pu) or 24GLYE7 (24G) virus overcame the drug selection quickly, and rapid proliferation of the culture was evident within 3 days. In contrast, the number of viable cells in DIN cultures infected with wt E7 virus remained extremely low for a long period, despite the presence of IL-3. When the E7-infected cells eventually reached a proliferative stage and a growing puromycin-resistant population could be established, the amount of

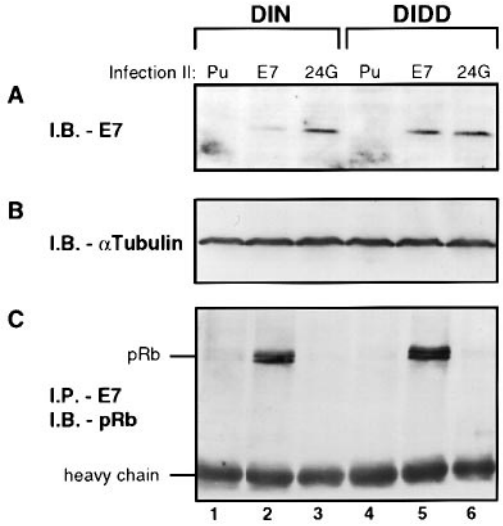

Fig. 4. Western blot analysis of double-infected populations derived from DA-1 cells (Figure 3). Total protein extracts were made from newly established puromycin-resistant populations; the viruses used for the second infection are indicated on top and are designated as in Figure 3. Extracts were either subjected directly to Western blot analysis with anti-E7 antibodies (**A**) or anti-α-tubulin antibodies (**B**), or first immunoprecipitated (I.P.) with anti-E7 antibodies followed by Western blot (I.B.) analysis with anti-pRb antibodies (**C**).

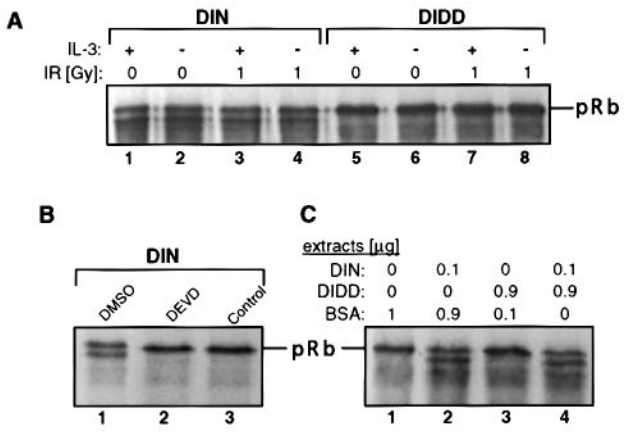

Fig. 5. *In vitro* cleavage of pRb. (**A**) *In vitro* translated ^{35}S-labeled pRb was mixed with 1 μg of protein extract from DIN (lanes 1–4) or DIDD (lanes 5–8) cells. Before extraction, cells were maintained under different conditions as indicated above each lane; irradiation (IR) was performed 3 h before extraction and IL-3 deprivation was for 9 h. Following incubation for 30 min at 37°C (see Materials and methods), the ^{35}S-labeled pRb products were analyzed using SDS–PAGE followed by fluorography and autoradiography. (**B**) Reactions were performed as in panel A, employing extracts of untreated DIN cells (equivalent to lane 1, Figure 5A), in the presence of the caspase inhibitor DEVD-fmk (100 μM) or of DMSO, used for controlling solvent effects. In lane 3, no cell extract was included. (**C**) Extracts prepared from DIN or DIDD cells maintained under normal culture conditions were mixed in the indicated combinations and pRb cleavage was assayed as above.

E7 protein in this population was found to be very low (Figure 4A, lane 2). This suggests a strong selection against cells that were successfully infected and expressed efficiently the wt E7 protein. Unlike DIN cells, DIDD emerged rapidly from selection following infection with the wt E7 virus (Figure 3B); as expected, no significant differences between DIN and DIDD were observed with the control viruses (Figure 3C and D). Moreover, the amount of wt E7 protein in the growing cultures of infected DIDD cells was much higher than in DIN (Figure 4A, compare lane 5 with lane 2), and comparable with that of the 24GLYE7 protein in both cell types (Figure 4A, lanes 3 and 6). As expected, the wt E7 protein in both cell types was capable of binding pRb, whereas 24GLYE7 was not (Figure 4C). These observations support the notion that pRb inactivation results in p53-dependent death of DA-1 cells even in the presence of IL-3.

p53 regulates the potential activity of a pRb-cleaving caspase(s)

The data presented above support a model where IL-3 withdrawal activates a caspase that cleaves pRb and accelerates apoptosis through a mechanism whose efficient execution requires functional wt p53. To further investigate the role of p53 in pRb cleavage, we employed an *in vitro* cleavage assay. pRb was supplied exogenously as an [^{35}S]methionine-labeled product of an *in vitro* transcription/translation reaction. This pRb was mixed with extracts of DIN (Figure 5A, lanes 1–4) or DIDD (Figure 5A, lanes 5–8) cells, treated prior to extraction as indicated above each lane. Incubation with DIN cell extracts resulted in the appearance of a faster migrating form of pRb, implying that pRb can be cleaved under these conditions. Surprisingly, unlike the cleavage of endogenous pRb within intact cells, the extent of exogenous pRb cleavage *in vitro* was identical for all DIN extracts, irrespective of prior treatment or extent of apoptosis at the time of extraction (Figure 5A, lanes 1–4). Cleavage was blocked by DEVD-fmk (Figure 5B), confirming that the faster migrating pRb form was due to *in vitro* cleavage by a caspase. Longer incubation resulted in further degradation and complete disappearance of pRb (data not shown), mirroring the situation seen *in vivo* in the absence of IL-3. The fact that pRb cleavage occurred in DIN extracts irrespective of prior cell history suggests that the pertinent caspase(s) exists in a ready-to-go form in these cells even under normal culture conditions, but is kept inactive by the presence of IL-3. The extraction process presumably relieves this inhibition and renders the caspase active.

Importantly, extracts from DIDD cells, where p53 activity is compromised, were incapable of efficient pRb cleavage (Figure 5A, lanes 5–8). Thus, the potential activity of this caspase appears to be dependent largely on the presence of functional wt p53 within the cells.

The mechanism through which p53 regulates the potential activity of the pRb-cleaving caspase(s) is not known. It is possible that, even at low basal levels, p53 contributes an activity required for caspase activation. Alternatively, cells lacking p53 may express an inhibitor of caspase activation. To address these possibilities, we tested whether an inactive extract can be activated by an active one, or alternatively whether it can inhibit the active extract. When a DIDD extract was mixed with a DIN cell extract at a ratio of 9:1, pRb cleavage by the mixture was identical to that seen with the active component alone (Figure 5C, compare lane 4 with lane 2). Hence, the pRb cleaving activity of the DIDD extract can not be activated *in vitro* by addition of a DIN extract, and the DIDD extract can not exert any caspase-inhibitory effect.

Discussion

In DA-1 cells, p53 contributes to efficient death upon IL-3 deprivation (Gottlieb *et al*., 1994). Yet excess wt p53 activity on its own is insufficient to cause apoptosis as long as the cells remain exposed to IL-3. Hence it is possible that additional proteins, whose expression or function are modulated by survival factors, will dictate the cellular outcome of p53 activation. The data presented here suggest that pRb, a tumor suppressor gene product, is one of these proteins. Thus withdrawal of IL-3 promotes caspase-mediated cleavage of pRb, presumably leading to its eventual inactivation. As also suggested by the effect of the HPV E7 oncoprotein, a potent pRb inactivator, loss of pRb function renders DA-1 cells more likely to die in the presence of functional p53. Interestingly and perhaps surprisingly, p53 itself also appears to play an important role in controlling pRb cleavage, as best illustrated by the *in vitro* degradation assays.

pRb can modulate the cellular outcome of p53 activity and prevent apoptosis

The pRb protein is a cardinal contributor to the cell-cycle inhibitory effects of p53. One way pRb participates in controlling the G_1–S-phase transition is by binding to and regulating the activity of E2F family transcription factors (Wang, 1997). pRb also binds other proteins, such as Mdm2 (Xiao *et al*., 1995) and c-Abl (Wang, 1997); binding of the latter may also be crucial for pRb's growth suppression activity (Wang, 1997).

Aside from its ability to regulate cell growth, numerous observations also suggest that pRb has a role in inhibition of apoptosis. Cells deficient in pRb function appear prone to excessive death, as shown in *Rb–/–* mouse embryos (Clarke *et al*., 1992; Jacks *et al*., 1992; Lee *et al*., 1992; Morgenbesser *et al*., 1994; Macleod *et al*., 1996; Zacksenhaus *et al*., 1996). Increased p53-mediated apoptosis after DNA damage is seen in fibroblasts lacking the *Rb* gene (Almasan *et al*., 1995). Expression of viral oncoproteins that bind to and inactivate pRb, including the HPV E7 protein, augments apoptosis both in whole animals (Fromm *et al.*, 1994; Howes *et al*., 1994; Pan and Griep, 1994) and in cultured cells (Debbas and White, 1993; Hansen *et al*., 1995; Slack *et al.*, 1995). It is important to note that these viral oncoproteins bind additional cellular proteins, including p107 and p130. However, unlike the case for pRb, the relationship of those other proteins to apoptosis remains unclear. In a more direct approach, induction of apoptosis was seen in epithelial cells overexpressing a putative dominant negative pRb mutant (Whitaker and Hansen, 1997). pRb dysfunction thus appears to promote apoptosis, and in certain circumstances this effect is dependent on the presence of functional wt p53 (White, 1994). This notion is also supported by the E7 experiments described in Figure 3. pRb overexpression was also shown to protect against apoptosis induced by a variety of stimuli including oncogene activation (Qin *et al*., 1994), DNA damage (Haas-Kogan *et al*., 1995), p53 overexpression (Haupt *et al*., 1995b), interferon-γ (IFN-γ) (Berry *et al*., 1996), transforming growth factor-β (TGF-β) (Fan *et al*., 1996) and ceramide (McConkey *et al*., 1996), as well as during myocyte differentiation (Wang *et al*., 1997).

A possible link between pRb and p53 in the regulation of apoptosis is E2F-1. E2F-1-deficient mice reveal a block in thymocyte apoptosis during development (Field *et al*., 1996). Loss of a pro-apoptotic function of E2F-1 may account for the surprising finding that such mice display increased cancer susceptibility (Field *et al*., 1996; Yamasaki *et al*., 1996). Furthermore, when overexpressed in cultured cells, E2F-1 can cooperate with p53 to induce apoptosis (Qin *et al*., 1994; Shan and Lee, 1994; Wu and Levine, 1994). In the IL-3-dependent myeloid cell line 32D, overexpression of E2F-1 together with its dimeric partner DP-1 results in rapid apoptosis (Hiebert *et al*., 1995). Remarkably, as with E7 (Figure 3), the apoptotic activity of E2F-1+DP-1 also appears to involve p53 and can not be overcome by IL-3 (Hiebert *et al*., 1995). Therefore, the anti-apoptotic effect of pRb may be achieved, at least in part, through E2F-1 inhibition.

pRb cleavage and its relationship to apoptosis

We provide evidence that in DA-1 cells exposed to apoptotic stimuli, pRb is cleaved by a member(s) of the caspase family of cysteine proteases. Caspases are important mediators of apoptosis (reviewed in Cohen, 1997; Miller, 1997; Salvesen and Dixit, 1997). pRb cleavage in DA-1 cells is regulated positively by p53 and negatively by a survival factor, IL-3. This cleavage results in the eventual elimination of pRb, and is correlated with the induction of rapid p53-dependent apoptosis instead of a p53-dependent growth arrest.

pRb was shown recently to be a caspase substrate, and there exists a correlation between pRb cleavage and various apoptotic responses (An and Dou, 1996; Janicke *et al.*, 1996; Chen *et al*., 1997; Tan and Wang, 1998). Apoptosis-associated pRb cleavage involves the removal of 42 C-terminal residues (Janicke *et al*., 1996; Chen *et al*., 1997; Tan *et al*., 1997; Tan and Wang, 1998). A point mutation within the predicted caspase cleavage site blocks pRb proteolysis both *in vitro* (Janicke *et al*., 1996) and *in vivo* (Tan *et al*., 1997; Tan and Wang, 1998). Importantly, a non-cleavable pRb mutant antagonizes tumor necrosis factor (TNF)-induced apoptosis (Tan *et al*., 1997; Tan and Wang, 1998). This indicates that pRb cleavage is not only associated with, but is actually required for, certain types of apoptosis.

Here IL-3, a potent survival factor, is shown to prevent pRb cleavage in intact cells. This inhibitory effect also occurs in the absence of p53 function (Figure 1A, lane 2), but is significantly enhanced in cells which possess functional wt p53. The mechanism through which IL-3 prevents pRb cleavage is unknown at present. However, it may depend on the phosphorylation of the pro-apoptotic protein BAD, resulting in its cytoplasmic sequestration and inactivation (Gajewski and Thompson, 1996; Wang *et al*., 1996; Zha *et al*., 1996). In turn, this may free anti-apoptotic members of the bcl2 family from the inhibitory effects of BAD and allow them to prevent caspase activation (Reed, 1997).

Caspase-mediated pRb cleavage may be only the first step towards complete degradation of pRb, a conjecture supported by the decrease in overall pRb concentration in DA-1 cells deprived of IL-3 (Figure 1B). Alternatively, the mere truncation of pRb may already suffice to eliminate its anti-apoptotic effects. A third possibility is that the

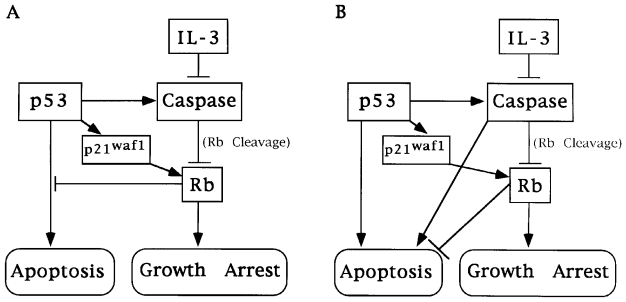

Fig. 6. Schematic model for the roles of p53 and pRb in apoptosis of DA-1 cells after IL-3 withdrawal. Two variations are depicted. It is proposed that pRb can block p53-mediated apoptosis, but this activity of pRb is eliminated by a caspase which is regulated positively by p53 and negatively by IL-3. The IL-3/p53-dependent caspase(s) may be required exclusively for pRb cleavage (**A**), or also for cleavage of additional substrates as part of the actual apoptotic process (**B**).

truncated form of pRb might have an active positive role in regulating the death machinery; however, overexpression of exogenous pRb lacking the last 42 amino acids does not potentiate TNF-induced apoptosis (Janicke *et al*., 1996). Hence, it is possible that pRb cleavage contributes to apoptosis by alleviating an apoptosis-inhibitory effect of intact pRb.

It is notable that two other members of the pRb family, p107 and p130, are not cleaved during DA-1 apoptosis (data not shown). Moreover, the defined cleavage site in pRb (Janicke *et al*., 1996; Chen *et al*., 1997) is not present in these two proteins. Thus it appears that these members of the family, while capable of interaction with the cell-cycle machinery, may not to be involved in regulation of apoptosis. Indeed, mice carrying a null mutation for *p107* or *p130* do not exhibit accelerated apoptosis (Cobrinik *et al*., 1996; Lee *et al*., 1996), unlike *Rb*-knockout mice.

The potential activity of the pRb-cleaving caspase is regulated by p53

We propose a new role for p53 in the regulation of apoptosis in DA-1 cells. It is suggested that p53 regulates the potential activity of a caspase(s) which cleaves pRb (and probably additional substrates). The major observable biochemical effect of p53 inactivation, through use of a negative dominant p53 mutant (DD), relates to the ability of this caspase to become active *in vitro* (Figure 5A). The basal function of endogenous wt p53 is therefore required, albeit not sufficient *in vivo*, for activation of this caspase. The actual activity of this caspase within the cell is controlled by an external signal, IL-3. The presence of functional p53 activity thus predisposes DA-1 cells to apoptosis, rendering them subject to tight regulation by survival factors. As discussed above, the consequence of pRb cleavage is facilitation of cell death. However, this death is in itself p53-dependent, as demonstrated by the observation that pRb inactivation by E7 expression is not enough to induce apoptosis when p53 is non-functional (in DIDD cells).

In summary, p53 can modulate apoptosis both by affecting the activity of pRb, an inhibitor of apoptosis, and by positive regulation of the apoptotic machinery itself, including a variety of pro-apoptotic p53 target genes. Figure 6 depicts two versions of the proposed model. It remains to be established whether the particular IL-3/p53-regulated caspase(s) is dedicated exclusively to pRb cleavage (Figure 6A), or also plays a critical role in other distinct stages of the apoptotic process, through cleavage of different protein substrates (Figure 6B). Caspases are necessary for p53-mediated apoptosis (Sabbatini *et al*., 1997). In the study by Sabbatini *et al*. (1997), caspase activation and apoptosis were due to Bax induction as a consequence of p53 SST activity. Although pRb cleavage was not reported to occur in these cells, it is of note that pRb was inactivated by the adenovirus E1A oncoprotein. Since in DA-1 cells *bax* is not induced after IL-3 withdrawal (Gottlieb *et al*., 1996), it is possible that p53 acts upstream of several caspases, some induced by IL-3 withdrawal and others through Bax activation. The model depicted in Figure 6B therefore appears more realistic; p53-mediated caspase activation can occur in several ways, probably involving multiple caspases, all of which are required for an optimal apoptotic response. Needless to say, this model relates only to the p53-dependent aspects of caspase activation during apoptosis. Caspases also play critical roles in p53-independent apoptosis, and thus can also be activated by mechanisms which do not rely on p53.

In conjunction with earlier work by others, our data place pRb at an important decision fork for the biological consequences of p53 activity. Moreover, they suggest a mechanism for the action of IL-3 as a survival factor. IL-3 can regulate the decision between p53-mediated growth arrest and apoptosis (Canman *et al*., 1995; Gottlieb *et al*., 1996), and the same may hold for several other survival factors (Abrahamson *et al*., 1995; Lin and Benchimol, 1995). Interestingly, IL-3 and IL-2 are capable of controlling the activity of caspase-3 (CPP32) (Ohta *et al*., 1997); furthermore, IL-6 was also found to block caspase activation during apoptosis induced by p53 or other mediators (Lotem and Sachs, 1997). It remains to be investigated whether regulation of pRb cleavage also contributes to the effect of survival factors in other biological systems.

Materials and methods

Plasmids and retroviruses

Plasmids pLXSNp53val135 and pLXSNp53DD were used to produce retroviruses encoding the ts mutant p53val135 and the dominant negative C-terminal fragment of p53, DD, respectively (Gottlieb *et al*., 1994), whereas the parental pLXSN plasmid was used to produce control virus. These viruses were produced with the help of the packaging cell line GP+E-86. All viruses also confer G418 resistance.

The cDNAs of HPV type 16 wt E7 and mutant E724GLY were excised from plasmids pSP65/16E7 and pSP65/16E724GLY, respectively (Edmonds and Vousden, 1989). *Sal*I–*Eco*RI fragments from these plasmids were ligated into pBabe-puro (Morgenstern and Land, 1990), thereby generating pBabe-puro/16E7 and pBabe-puro/16E724GLY; all plasmids confer puromycin resistance. These plasmids as well as parental pBabe-puro were used to produce recombinant retroviruses by transient co-transfection together with a ψ^- ecotropic packaging vector, pSV-ψ^-–E-MLV (Muller *et al*., 1991) into 293T cells (Pear *et al*., 1993). Viral stocks were collected every 8 h for a total of 48 h. Working stocks (pooled collections) were kept temporarily at 4°C and then aliquoted and stored at –80°C.

DA-1 cells were infected with the neo-resistance viruses by co-cultivation with virus-producing cells as described before (Gottlieb *et al*., 1994). This gave rise to cell pools expressing the neo-resistance gene either alone (DIN) or together with the ts p53val135 mutant (DIV) or the dominant negative C-terminal fragment of p53 (DIDD).

Infection of DIN and DIDD cells with puromycin resistance viruses

was carried out as follows: 1.5 ml of virus-containing stock was added to 10^5 DIN or DIDD cells in the presence of IL-3 and 8 μg/ml of polybrene (Sigma) and cells were incubated for 5 h at 37°C. Then, 4 ml of fresh medium were added. The next day, the cultures were replenished with fresh medium containing 3 μg/ml puromycin (Sigma) and maintained in presence of the drug until a proliferating pool of cells was obtained.

Cell culture and viability assays

Cells were maintained routinely at 37°C in DMEM supplemented with 10% fetal calf serum (FCS) and 50 μM β-mercaptoethanol. DA-1 cells and their derivatives were supplemented with 0.1% medium conditioned by X63/0 cells (Karasuyama and Melchers, 1988), serving as a source of IL-3. For factor-withdrawal experiments, cells were washed twice by pelleting and resuspension in IL-3-free medium, and then resuspended in fresh medium with or without IL-3 at a density of 2×10^5 cells per ml. Where appropriate, DIV6 cells were transferred to 32°C immediately after being washed and resuspended. Irradiation (by a γ source of ^{60}Co) of DIN or DIDD cells was performed 3 h after the cells were washed and resuspended. Viability was determined by trypan blue exclusion.

To block caspase activity, the modified tetrapeptide caspase inhibitor Asp-Glu-Val-Asp-fluoromethylketone (DEVD-fmk; 200 μM final concentration) was added to the culture immediately after washing and resuspension. DMSO was used to control for solvent effects.

Protein analysis

pRb was detected by probing Western blots with monoclonal antibody G3-245 (Pharmingen), and HPV type 16 E7 protein was detected with a specific monoclonal antibody (Triton diagnostics). For coprecipitation assays, 1 mg of protein extract was immunoprecipitated with anti E7 polyclonal antibodies (kindly provided by Dr K.Vousden) immobilized to Protein A resin (Sigma). Proteins were eluted from the resin by boiling in sample buffer (50 mM Tris pH 6.8, 2% SDS, 2% β-mercaptoethanol and 10% glycerol). Eluates were subjected to SDS–PAGE followed by Western blot analysis with anti-pRb antibodies. Blots were developed with the ECL-enhanced chemiluminescence system (Amersham).

In vitro pRb cleavage assays

Cells were extracted in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) supplemented with 5 mM NaF, 0.5 mM sodium vanadate, 60 U/ml Aprotinin (Calbiochem) and 0.3 mg/ml PMSF. The protein concentration was determined using the Bradford reagent (Protein assay, Bio-Rad). The reaction was performed as follows: 1 μg of protein extract (1 mg/ml stock concentration) was mixed with 1 μl of the *in vitro* translation reaction (TnT, Promega) containing ^{35}S-labeled pRb, in 20 μl of reaction mixture (20mM HEPES pH 7.5, 10% glycerol, 2.5 mM DTT, 5 mM NaF, 0.5 mM sodium vanadate). The reaction was incubated at 37°C for 30 min and terminated by adding sample buffer and boiling immediately. Products were analyzed by SDS–PAGE. Gels were fixed in 10% acetic acid for 1 h. Fluorography was performed by soaking the gel in 1 M sodium salicilate for 30 min , prior to gel drying and exposure to X-ray film.

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