[Frontiers in Bioscience 3, d419-430, April 16, 1998]

RB AND APOPTOTIC CELL DEATH

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Received 4/2/98 Accepted 4/6/98

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1. ABSTRACT

Homeostasis of cell numbers is achieved by balancing the proliferative and death states of cells. Proper regulation in a cell requires an accurate coordination between these two processes. Indeed, dysregulation of cell cycle progression is essential for the initiation of apoptosis. Retinoblastoma protein (RB) is an important tumor suppressor and a cell cycle regulator. Most recent studies suggest that RB also plays a regulatory role in the process of apoptosis. During the onset of apoptosis, the hyperphosphorylated form of RB (p120/hyper) is converted to a hypophosphorylated form (p115/hypo), which is mediated by a specific protein-serine/ threonine phosphatase activity. Accompanied by the internucleosomal fragmentation of DNA, the newly formed p115/hypo/RB is immediately cleaved by a protease that has properties of the caspase family. During apoptosis, RB is also cleaved in its carboxyl terminus by a caspase-3-like activity. By contrast, the unphosphorylated form of RB (p110/unphos) remains uncleaved during apoptosis. Further studies suggest that p110/unphos/RB functions as an inhibitor of apoptosis. Therefore, regulation of the RB proteolytic activities and consequent RB levels is important for the determination of cellular fate.

2. INTRODUCTION

Cell numbers are regulated by a balance between proliferative and anti-proliferative states of cells. Anti-proliferative states include differentiation, growth arrest, senescence (cellular aging), and apoptosis (programmed cell death). Proliferation in normal mammalian cells is tightly controlled in the late G_1 phase of the cell cycle through a process that involves cyclins, cyclin dependent kinases (CDKS), CDK inhibitors (CDKIS), retinoblastoma (RB), and other regulatory proteins (1, 2).

Apoptosis plays an important role in physiological processes, such as immune- and nervous-system development (3-6), and contributes to defense mechanisms important for the prevention of infectious illness and cancer (7-9). Apoptosis is an active, energy-dependent process of cellular self-destruction that involves shrinkage of cytoplasmic volume, membrane blebbing, chromatin condensation and chromosomal DNA fragmentation (10). Apoptosis can be triggered by various external stimuli including DNA-damaging agents such as chemotherapeutic drugs and irradiation (11-13).

Recent evidence suggest that intracellular signals involved in regulating cell proliferation and cell cycle progression also mediate apoptosis (11-13). For example, the tumor suppressor retinoblastoma (RB) protein has been shown to play important roles in the processes of cell proliferation, the G_1 to S phase transition, differentiation and senescence (see below). More recent studies also suggest involvement of RB in regulating apoptotic cell death (14). In this review, we will first summarize the functions of RB in other cellular processes and then review potential involvement of RB in the regulation of apoptosis.

2.1. RB as a tumor suppressor and a cell cycle regulator

The RB protein is the product of the retinoblastoma susceptibility gene Rb-1, which is the first cloned tumor suppressor gene that has growth inhibitory activity (15-17). The following evidence support the notion that RB is a tumor suppressor protein. First, in retinoblastomas, small cell lung carcinomas, bladder carcinomas and many sarcomas, RB function is lost through gene mutations. Secondly, introduction of the wild-type Rb gene into retinoblastoma-negative ($Rb^{-/-}$) tumor cells inhibited the formation of tumors in nude mice. Thirdly, mice with heterozygous Rb mutations ($Rb^{+/-}$) develop tumors at a high frequency. Finally, although the wild-type Rb gene is present in some human cancers, the RB tumor suppressor

function has been inactivated in these cancers by different mechanisms (15-17). For example, the great majority of cervical carcinomas express the human papillomavirus (HPV) E7 oncoprotein that binds to RB and inactivates its function. Many esophageal, breast, squamous cell carcinomas, glioblastomas, and B cell lymphomas contain amplified levels of an RB kinase component such as cyclin D or cdk4 gene, resulting in phosphorylation of RB and consequent inactivation of its tumor suppressor function. Many esophageal squamous cell carcinomas, glioblastomas, lung, bladder, and pancreatic carcinomas do not express the gene encoding an RB kinase inhibitor (CDKI, such as p16/INK4A or p15/INK4B), leading to constitutive activation of RB kinases which phosphorylate and inactivate RB (15-17).

In addition, RB has been found to play an important role in the process of the G_1 to S transition. This RB function is regulated by its phosphorylation status. Although the level of RB protein does not vary during the cell cycle progression, its phosphorylation status undergoes cyclical changes. RB is un-phosphorylated or hypo-phosphorylated in G_0 and early G_1 phases of the cell cycle. It becomes first phosphorylated around the middle of G_1 phase by cyclin D/CDK4 and cyclin D/CDK6 kinases, and further phosphorylated in the late G_1 by cyclin E/CDK2 kinase, in S phase by cyclin A/CDK2 kinase, and in G_2 and M phases by cyclin B/CDC2 kinase (18, 19). Phosphopeptide analysis demonstrates that RB is phosphorylated on more than a dozen distinct serine or threonine residues during the cell cycle progression (15-17). When cells re-enter G_1 from M phase for another cycle, RB becomes dephosphorylated by an activated type 1 protein-serine/ threonine phosphatase activity (PP1) (20, 21).

It has been hypothesized that it is the un- or hypo-phosphorylated, but not the hyper-phosphorylated, form of RB that has the growth inhibitory function. This hypothesis is supported by the following evidence. First, only the un- or hypo-phosphorylated form of RB binds to, and inactivates the cellular transcription factor E2F (22). Secondly, only the un- or hypo-phosphorylated form of RB binds to DNA viral oncoproteins, such as HPV16 E7, adenovirus E1A, and simian virus 40 (SV40) large T. Binding of RB to DNA viral oncoproteins results in inhibition of RB tumor suppressor function and up-regulation of cell growth (15-17). Thirdly, introduction of the Rb gene into the human osteogenic sarcoma cell line SAOS-2, which lack full length nuclear RB protein, produced high levels of un-phosphorylated form of RB (p110/unphos/RB) and arrested these cells in G_0/G_1 phase in a metabolically active state, while co-transfection of cyclin D2, E or A resulted in RB hyper-phosphorylation and overrode the G_1 block (23, 24).

Probably the best characterized RB target protein is the transcription factor E2F. The hypothesis that RB regulates the E2F function is supported by the following evidence. First, ectopic expression of wild-type Rb gene in Rb-negative cells causes a 5-fold decrease in the activity of a promoter containing E2F-binding sites, whereas expression of a mutant RB protein, which can not bind to E2F, has no inhibitory effect on the E2F-driven promoter activity. Secondly, transactivation studies using E2F-1 fusion proteins demonstrate that expression of RB inhibits the transcriptional activity of the fusion proteins containing wild-type E2F-1 but not transcriptionally active E2F-1 mutants (15-17, 22). Thirdly, the un- or hypo-phosphorylated form of RB is capable of binding to E2F (specifically, E2F-1, E2F-2 and E2F-3), resulting in inhibition of the E2F-mediated transactivation. When RB is phosphorylated, it can no longer bind to E2F. The free E2F, therefore, activates transcription of some cell growth control genes, including thymidine kinase, dihydrofolate reductase, c-myc and cdc2 (15-17, 22, 25).

In vitro binding studies, using both naturally occurring and artificially produced RB mutations, have defined several distinct protein-binding domains in RB. The first identified protein-binding region in RB consists of two nonconsecutive stretches of amino acids, called domain A (amino acids 379-572) and domain B (amino acids 646-772). These two domains, separated by an insert domain (amino acids 573-645), are thought to form a protein binding "pocket" termed the A/B pocket (15-17). The A/B pocket has been shown to interact with several viral oncoproteins (E7, E1A and large T antigen) and cellular nuclear proteins (including the transcription factor E2F) (15-17, 22). Furthermore, many of the naturally occurring RB mutations in human cancers are found in the A/B pocket region (15-17). A number of these point mutations resulted in the deletion of entire exons during RNA splicing. Other point mutates generate missense mutations, preventing the proper folding of the A/B pocket (26). Another protein-binding domain in RB is the C terminal pocket (amino acids 768-928), which functions independently of the A/B pocket. The binding of c-Abl oncoprotein to RB through the C pocket (27) is different from all previous known RB binding mechanisms because it is not affected by the viral oncoproteins, which displace proteins bound to the A/B pocket.

2.2. RB as a regulator of cellular differentiation and senescence

In addition to its function as a cell cycle regulator, RB has also been shown to play an essential role in the process of differentiation. For example, mouse embryos that are homozygous for mutated Rb-1 develop abnormalities in neurogenesis and erythropoiesis, leading to embryonic cell death around day 15 of gestation (28-30). Histological observations in these studies suggest a role for the RB protein in terminal differentiation. Consistent with the hypothesis that RB functions as a regulator for differentiation, the level of RB protein is greatly increased during differentiation in SCID (severe combined immunodeficiency) mouse fetuses (31). In addition, RB becomes dephosphorylated and activated prior to cell growth arrest during differentiation of human leukemic cells (32). Furthermore, un- or hypo-phosphorylated RB forms a complex with the myogenic factor MyoD, mediating transcription of muscle-specific genes (33).

These data suggest that RB is involved in the commitment of cells to differentiate.

It appears that the inhibition of CDK kinases is responsible for differentiation-associated RB dephosphorylation. It has been found that when myeloid stem cells are induced to differentiate into neutrophils, the levels of both CDK4 and D cyclins are down-regulated (34). In addition, upon induction of differentiation in murine erythroleukemia cells, the levels of CDK4 protein greatly decrease (35). Furthermore, the levels of p21/Waf-1, a CDK inhibitor, increase dramatically during differentiation of human leukemic cells (36). Another CDK kinase inhibitor p27 has been reported to be accumulated in the beginning of differentiation (37). Overexpression of an RB kinase component (such as CDK4, cyclins D2 or D3) prevents the differentiation process (34, 35). Interaction of the unphosphorylated form of RB with the helix-loop-helix protein Id-2, a cellular differentiation inhibitor, stimulates cellular proliferation (38). Therefore, interactions of RB with its cellular target proteins may function as a switch between proliferation and differentiation.

Human diploid fibroblasts (HDF) undergo replicative senescence predominantly because of cell growth arrest at the G_1/S boundary of the cell cycle (39). Previous studies have suggested that senescent HDF contain an inhibitor for the S phase entry (39). Recent studies have demonstrated that senescent cells contain high levels of unphosphorylated form of RB which acts as an endogenous inhibitor for S phase entry (40, 41). Further experiments have demonstrated that failure of senescent cells to phosphorylate RB is due to failure to activate RB kinases. Indeed, senescent cells contain high levels of inactive cyclin D1/CDK2 and cyclin E/CDK2 kinase complexes (42) and high levels of the CDK inhibitors such as p21 (43) and p16 (44, 45). Failure to activate RB kinases, therefore, may be responsible for the inability of senescent cells to phosphorylate RB in late G_1 phase, which in turn inhibits E2F-mediated transcription of genes required for entry into S phase.

3. INTERIOR CLEAVAGE OF RB DURING APOPTOSIS

3.1. Activation of caspases during the initiation of apoptotic execution

Apoptosis occurs in two physiological stages, commitment and execution (11-13, 46). Although little is known about the molecular controls of apoptotic commitment in mammalian cells, it has been suggested that cell cycle checkpoint regulators, such as p53 in some cell systems (2), might be involved in committing a cell to apoptotic death. The apoptotic execution is initiated by activation of specific proteases of caspase family, which exhibit a rather unique substrate specificity, cleavage after Asp residues (46). To date, at least ten homologs of caspases have been identified and cloned (47). Activation of caspases leads to apoptosis probably through the proteolytic cleavage of several important cellular proteins. An increasing number of proteins have been shown to be cleaved during the apoptosis by the caspases. These proteins include poly(ADP-ribose) polymerase (PARP) (48, 49), the 70 kDa component of the U1 small ribonucleoprotein (U1-70 kDa) (50), lamins (51), actin (52), sterol regulatory element-binding proteins (53), DNA-dependent protein kinase (54), protein kinase C-delta (PKC-delta) (55), PKC-theta (56), PKC-related kinase 2 (57) and RB (see below). These caspase-mediated cleavages can be blocked by expression of the cowpox virus CrmA protein or the Bcl-2 oncoprotein and also by a tetrapeptide mimics of the substrate cleavage site, such as acetyl-Asp-Glu-Val-Asp-fluoromethyl-ketone (DEVD-FMK) (49-57).

Although multiple protein substrates of caspases have been found, the functional significance of the cleavages is poorly understood. Since Lamins and actin are proteins functioning in cell structure and PARP, U1-70 kDa, sterol regulatory element-binding proteins and PKC are catalytic proteins involved in homeostatic pathways, it has been proposed that degradation or cleavage of these cellular proteins may abolish the critical structures and functions of the cells, leading to apoptosis. Most recent reports also suggest that some caspase cleavage products may be able to induce apoptosis. For example, Bcl-2, an integral intracellular membrane protein with an apoptosis-inhibiting activity, can be cleaved at Asp34 by caspase-3/CPP32 (58). Expression of the carboxyl terminal Bcl-2 cleavage product was able to trigger or accelerate apoptotic cell death (58). PKC-delta is also cleaved by caspase-3 at Asp354 site and subsequently activated. Overexpression of the cleaved, active PKC fragment, but not the full-length protein, resulted in induction of sub-G_1 phase DNA, nuclear fragmentation, and lethality (55).

3.2. Dephosphorylation of RB at an early stage of apoptosis: production of a caspase substrate

We have found that during the process of apoptosis, RB becomes first dephosphorylated and immediately cleaved (59, 60). Exponentially growing HL-60 cells contain mainly two forms of RB protein with apparent molecular masses 120 (p120/hyper) and 110 kDa (p110/unphos), respectively, detected by a purified monoclonal RB antibody G4-340. When the HL-60 cell extract was treated with alkaline phosphatase *in vitro*, the level of p120/hyper was decreased and that of p110/unphos was increased, indicating that p120 is the hyper-phosphorylated while p110 is the un-phosphorylated form of RB. The p120/hyper, but not the p110/unphos, form of RB was also detected by two other monoclonal antibodies, XZ55 and G3-245 (59).

When HL-60 cells were treated with a number of anti-cancer drugs, such as cytosine arabinoside (Ara-C) and etoposide (VP16), for 3 to 4 h, the level of p120/hyper/RB decreased and, simultaneously, a new, abundant band of 115 kDa (p115/hypo) was observed, detected by all the three RB antibodies (59). This result indicates that RB was dephosphorylated by the treatment of anti-cancer drugs.

Alkaline phosphatase treatment of the cell extract decreased the level of p115/hypo/RB, indicating that the p115/hypo/RB is partially but not completely dephosphorylated.

We also observed the early apoptosis-associated RB dephosphorylation in several other human leukemia cell lines including U937 and Jurkat T, which had been treated with various apoptotic stimuli, such as different anticancer agents (59, 60), an agonistic anti-Fas monoclonal antibody (61), a synthesized specific CDK inhibitor (roscovitine) (62), a PKC inhibitor (sphingosine or staurosporine) (62), and a proteasome inhibitor (unpublished data). Furthermore, when human breast cancer cell lines MCF-7 (containing the wild-type p53 gene) or MDA-MB-231 (containing a mutant p53 gene) were treated with VP-16 or tamoxifen, RB again became dephosphorylated prior to apoptosis (unpublished data).

Several other research groups have also reported apoptosis-associated RB dephosphorylation by using different cell systems or different apoptotic stimuli. When human Burkitt lymphoma cells were treated with ionomycin or an antibody to cell surface immunoglubin, RB dephosphorylation occurred rapidly even prior to cell shrinkage and chromatin condensation (63). Treatment of ML-1 myeloid leukemia cells (containing wild-type p53 gene) with VP-16 resulted in RB dephosphorylation prior to the internucleosomal DNA fragmentation (64). Treatment of human hepatoma HuH-7 cells with TGF-beta1 also induced RB dephosphorylation and apoptosis (65). All these results indicate that RB dephosphorylation is a common event in an early stage of apoptosis.

3.3. Cleavage of p115/hypo/RB into p68 and p48 fragments

After treatment of HL-60 cells with Ara-C for 4 h, a polypeptide band of 68 kDa (p68/RB) was detected by the RB antibody XZ55, while another p48/RB band was detected by antibody G3-245 (60). After 6 to 8 h treatment, the levels of p68/RB and p48/RB increased, which was associated with a decrease in the level of p115/hypo/RB. The 180-base-pair (bp) DNA ladder was also produced with kinetics similar to that of the p68 and p48 fragments of RB (60). These results indicate that p115/hypo/RB is cleaved into p68 and p48 fragments and that the interior cleavage of RB is tightly associated with the initiation of apoptotic execution. We have also found the interior cleavage of RB in human Jurkat T cells treated with roscovitine (a CDK inhibitor), sphengosine (a PKC inhibitor), staurosporine (a nonspecific PKC inhibitor), agonistic Fas antibody, or a proteasome inhibitor (61 and unpublished data), indicating that it is a common event in the process of apoptosis.

We have found that the p68/RB and p48/RB fragments have selective binding affinity to different RB antibodies. For example, only the p48/RB fragment is detected by RB monoclonal antibody G3-245 (60, 66) which reacts specifically with an epitope between amino acids 300-380 (67). In contrast, only the p68/RB fragment is detected by antibody XZ55, XZ77, XZ91 and XZ104 (60, 66), all of which recognize similar epitopes located in the A/B pocket (68). In addition, the p68/RB fragment was found in the high salt-extractable, nuclear fraction, while the p48/RB fragment was in the low salt-extracted fraction that contained mainly cytoplasmic proteins (66). All of these data point to the conclusion that the p48/RB fragment contains a sequence between the amino terminus and amino acid 443 of RB protein, whereas the p68/RB fragment consists of a sequence between amino acid 381 and the carboxyl terminus of RB that includes the A/B pocket region (amino acids 379-772).

The facts that p68/RB contains a sequence from the A/B pocket region and is only found in the nuclear fraction of the apoptotic cells suggest that p68/RB still has the ability to interact with cellular nuclear components. However, we have found that the nuclear binding partner of the p68/RB fragment is not the transcription factor E2F-1 since a specific E2F-1 antibody coimmunoprecipitated only the un-phosphorylated form of RB, but not the p68 fragment (66).

Since the induced hypo-phosphorylation of RB preceded its cleavage, it would appear that p115/hypo, rather than p120/hyper, is the substrate for the RB cleavage activity. Inhibition of the RB hypo-phosphorylation, therefore, should prevent the RB cleavage. Indeed, addition of a specific protein-serine/threonine phosphatase inhibitor, calyculin A, to the 1 h-Ara-C-pretreated HL-60 cells effectively prevented formation of both the p115/hypo and the p68 fragment of RB (60). This treatment also blocked the appearance of the apoptotic peak and production of the 180-bp DNA ladder (60). These data support the idea that the p115/hypo form of RB serves as the substrate for the RB interior cleavage enzyme.

3.4. Characterization of the RB interior cleavage activity

To characterize the RB interior cleavage activity, a variety of protease inhibitors were tested for their ability to prevent the interior cleavage process. Addition of iodoacedemide, a sulhydryl blocking reagent, at a final concentration of 50 micro M to cultures of HL-60 cells, that had been pretreated with Ara-C for 3 to 4 h, completely blocked generation of p68 and DNA ladder, and also increased the levels of p115/hypo and high molecular weight DNA fragments. However, addition of a chloromethyl ketone such as TLCK or TPCK had much less inhibitory effects on these drug-induced events. TLCK had no effects at 30 micro M on production of the p68 fragment of RB; only inhibited 50-60% of the product of the interior cleavage of RB at 200-400 micro M; and inhibited the interior cleavage of RB as well as the RB dephosphorylation only at >1 mM. Addition of other protease inhibitors, such as antipain, PMSF and phosphoramidon, had no inhibitory effects under experimental conditions (60).

We have noticed that the apoptosis-associated RB interior cleavage activity resembles that of the caspases responsible for cleavage of PARP (48, 49) and U1-70 kDa (50), which have been characterized both in cell cultures and in cell-free systems. All these three cleavage activities (RB, PARP and U1-70 kDa) were: (*a*) sensitive to treatment of iodoacedemide at a concentration of ~50 micro M, (*b*) much more resistant to TLCK at a concentration of 100-400 micro M, (*c*) blocked by TLCK only at 1 mM, and (*d*) resistant to treatment with other protease inhibitors such as antipain, PMSF and phosphoramidon (48-50, 60). These data suggest that a caspase activity is responsible for the interior cleavage of RB.

To provide additional evidence for this hypothesis, we used a well-characterized, specific tetrapeptide caspase inhibitor, acetyl-Tyr-Val-Ala-Asp-chloromethyl-ketone (YVAD-CMK) (69). This caspase inhibitor has the ability of effectively blocking some caspase activities in cell-free systems (49, 51) and preventing the Fas-mediated apoptosis in mouse W4 and human Jurkat cells (70). When incubated for 6 h with the 1 h-VP-16-pretreated HL-60 cells, YVAD-CMK effectively blocked production of the p68/RB and p48/RB, accompanied by the accumulation of p115/hypo/RB, again supporting the idea that the p115/hypo is the substrate for the RB interior cleavage enzyme. Treatment of the VP-16 pretreated cells with YVAD-CMK also prevented formation of DNA ladder, and simultaneously increased levels of high molecular weight DNA fragments (60). This data suggests that YVAD-CMK not only prevented the interior cleavage of RB, but also retained at least a portion of the drug-treated cells in an early apoptotic (but not the normal) stage. Addition of DEVD-FMK, a more specific inhibitor of caspase-3 (46), also blocked the RB interior cleavage and apoptosis (66).

Bcl-2 is the mammalian homologue of CED-9, which is a negative regulator of the cell death gene CED-3 (71). It has been shown that Bcl-2 and related family members inhibit apoptotic cell death induced by many stimuli (72, 73). CrmA is a protease inhibitor that prevents apoptosis by blocking one or more caspases (74). Expression of Bcl-2 in Jurkat cells completely prevented VP-16-induced RB cleavage and apoptosis (75). When Jurkat cells expressing CrmA were treated with VP-16 for up to 8 h, low levels of the RB cleavage products were detected, suggesting that CrmA, similar to Bcl-2, inhibits the interior cleavage of RB. Consistent with the partial inhibition by CrmA of VP-16-induced RB interior cleavage, low levels of DNA fragments were detected in these cells after 8 h of drug treatment (75). In the case of Fas agonistic antibody-induced apoptosis, CrmA was more potent than Bcl-2 at inhibiting RB proteolysis and apoptosis (61).

3.5. Comparison of PARP cleavage activity and RB interior cleavage activity

PARP is possibly the best characterized proteolytic substrate for caspases, being cleaved in the execution phase of apoptosis in many systems. PARP is cleaved at the sequence DEVDG by a caspase activity (49). At the present time, it appears that caspase-3 (CPP32) and caspase-7 (Mch3) are primarily responsible for PARP cleavage during apoptosis (76). *In vitro* experiments also showed that other caspases, including caspase-2, -4, -6, -8, -9 and -10, when added at high concentrations, can also cleave PARP or a synthetic substrate containing the DEVD sequence (76).

We compared the properties of the PARP and the RB interior cleavage activities. We have found that both cleavage activities were prevented by the caspase inhibitors, such as YVAD-CMK, DEVD-FMK, Bcl-2, and CrmA (66), consistent with that both PARP and RB are cleaved by caspases. However, the following evidence suggest that these two proteolytic activities are not identical. First, the RB interior cleavage activity was much more sensitive to YVAD-CMK and DEVD-FMK than the PARP cleavage activity (66). In addition, the RB cleavage occurred about 1 h after PARP cleavage in VP-16-treated Jurkat cells (unpublished data), indicating that those two proteases are activated at different times by drug treatment. Therefore, the RB interior cleavage activity is probably not the one that cleaves PARP.

3.6. Failure to induce the RB interior cleavage is associated with drug resistance

If the interior cleavage of RB is critical for induction of apoptosis by anti-cancer agents, failure to induce the interior cleavage of RB should be associated with drug resistance. To test this hypothesis, we used a pair of HL-60 lines which are sensitive or resistant to the anticancer agent Ara-C but both of which are sensitive to VP-16. Although both Ara-C and VP-16 are DNA-damaging agents, they act through different mechanisms. It has been suggested that cellular deoxycytidine kinase activates Ara-C, which inhibits DNA polymerase (77). In addition, incorporation of Ara-C into cellular DNA results in premature chain termination which may also count for the Ara-C-mediated cytotoxicity (78). In contrast, VP-16 blocks DNA replication by inhibiting the catalytic activity of topoisomerase II. More importantly, this inhibition leads to stabilization of the normally transient covalent intermediate formed between the DNA substrate and the enzyme (79).

Treatment of Ara-C sensitive HL-60 cells with Ara-C induced both the interior cleavage of RB and DNA fragmentation. When Ara-C-resistant HL-60 cells were treated with Ara-C, neither the RB cleavage nor DNA fragmentation were induced. In contrast, treatment of these Ara-C-resistant cells with VP-16 induced both the RB interior cleavage and DNA fragmentation, inhibitable by YVAD-CMK (80). Therefore, activation of the RB cleavage enzyme, a caspase protease, is required for overcoming drug resistance. Furthermore, the interior cleavage of RB correlated well with the activation of caspases. Ara-C treatment induced processing and activation of both caspase-1 (ICE) and caspase-3 (CPP32) only in sensitive (parental) HL-60, but not in Ara-C-

resistant cells. However, when Ara-C-resistant cells were treated with VP-16, both caspase-1 and caspase-3 were processed and activated (80). Our data indicate that activation pathways for caspase proteases are intact in these resistant cells. Therefore, activation of caspase pathway could be a novel strategy for the treatment of drug-resistant cancers.

4. CARBOXYL TERMINAL CLEAVAGE OF RB DURING APOPTOSIS

4.1. Production of an apoptosis-specific form of RB which migrates faster than its dephosphorylated form

In addition to dephosphorylation and interior cleavage, RB has also been found to be cleaved in its carboxyl (C) terminus during apoptosis (81-83). This was first observed by the appearance of an apoptosis-specific form of RB which migrates faster than the dephosphorylated form (~5 kDa smaller). When TNF-sensitive Hela D98 cells or HT-1080 fibrosarcoma cells were treated with a combination of TNF and cycloheximide (CHX), the hypo-phosphorylated RB was further converted to a faster migrating form, which was later found to be a C-terminal cleaved form of RB (81). Incubation of non-apoptotic Hela D98 cell extracts with calf intestinal phosphatase converted hyper-phosphorylated RB form to the hypo-phosphorylated form, but not to the specific C-terminal cleaved form of RB found in the apoptotic Hela D98 cells (81). In addition, when human Jurkat T cells were treated with Fas ligation, the hyper-phosphorylated RB bands were first converted to a series of hypo-phosphorylated bands and then to the C-terminal cleaved form (82). In HL-60 cells treated with VP-16 or Ara-C, not only the C-terminal cleaved form of RB but also the cleaved-off 5 kDa fragment were detected (83). The C-terminal cleavage of RB was also observed in cell lines treated with other different apoptotic stimuli, including exposure to staurosporine or cisplatin and withdrawal of serum (82). As expected, the C-terminal cleaved form of RB was recognized by RB antibodies against its N-terminus and the A/B pocket, but not by an antibody that detects the C-terminal 15 amino acid peptide, whereas the cleaved-off 5 kDa fragment was detected by the C-terminal antibody (81-83).

4.2. Characterization of the RB C-terminal cleavage activity

A caspase consensus cleavage sequence, DEADG (amino acids 883 to 887) (81-83), was found near the C-terminus of human RB, which is conserved in the mouse, chicken, and the *Xenopus* RB (82). This caspase consensus cleavage site is similar to the caspase-3 cleavage site on PARP (DEVDG) (49). Cleavage between Asp886 and Gly887 by a caspase would, therefore, remove a peptide of 42 amino acids (4.87 kDa) containing the C-terminal sequence of RB.

The following evidence suggest that generation of the C-terminal truncated RB is a result of cleavage at this caspase consensus site. First, the C-terminal cleavage of RB induced by TNF/CHX treatment was prevented by YVAD-CMK, a specific caspase protease inhibitor (81). In addition, generation of the C-terminal truncated RB by Fas ligation was inhibited by Z-VAD-CMK, an inhibitor of general caspases (82). Furthermore, granzyme B-induced C terminal cleavage of RB in an *in vitro* system was inhibited by CrmA or DEVD-aldehyde (82). Finally, an RB mutant with a truncation at the putative cleavage site migrated identically to the C terminal cleaved RB found in apoptotic cells (82). This deletion mutant, as well as another mutant with substitutions of Asp886 and Gly887 to Ala and Glu, respectively, were completely resistant to cleavage by granzyme B treatment. These data strongly suggest that RB is cleaved between Asp886 and Gly887 during apoptosis by a caspase protease.

4.3. Significance of the C-terminal cleavage of RB

It has been shown that induction of C-terminal truncated RB was tightly associated with induction of apoptosis, whereas inhibition of the C-terminal RB cleavage was associated with inhibition of apoptosis (81-83). The C-terminal cleavage of RB leads to the removal of the full length RB and the generation of both the C-terminal truncated RB and a 5 kDa peptide containing the C-terminal RB sequence. To assess if either of these two RB C-terminal products can directly induce apoptosis, one group transfected Hela D98 and MCF-7 cell lines with expressing constructs encoding the C-terminal truncated RB or the 5 kDa peptide of C-terminal RB sequence (81). Neither Hela D98 nor MCF-7 cells transfected with the construct encoding either C-terminal truncated RB or the 5 kDa peptide of C-terminal RB sequence showed enhanced apoptosis when compared with cells transfected with the vector alone (81). However, another group reported that when the non-degradable RB with a point mutation on the consensus cleavage site was expressed in $Rb^{-/-}$ 3T3 cells, TNF was no longer able to induce apoptosis (82). In all the cases, the decrease of the uncleaved, full-length RB correlated well with the increase in cell death induced by various stimuli (81-83). This is consistent with the known anti-apoptosis function of unphosphorylated form of RB (see Section 5).

Since the apoptosis inhibitory effect of full length RB may involve the binding of RB with the apoptosis-related proteins, such as E2F, cyclin D and MDM2, an altered affinity between the C-terminal truncated RB and those apoptosis-related proteins may be responsible for failure of RB to inhibit apoptosis. It has been shown previously that distinct domains on RB sequence are required to bind to different regulatory proteins. The E2F binding activity of RB requires both the A/B pocket domain and the C-terminal sequences (up to amino acid 849) (84). One group has found that the full-length RB and the C-terminal truncated RB have an equal efficiency of inhibiting E2F transcription activity (81). Another group has reported that the C-terminal truncated RB even has an enhanced binding affinity to E2F-1, E2F-3, and E2F-4 (83). Therefore, the C-terminal cleavage of RB does not decrease the E2F-1 binding activity. Releasing of free E2F should

not be responsible for the RB C-terminal cleavage-associated apoptosis.

Previous studies have demonstrated that cyclin D3 binds to the A/B pocket domain of RB (24). It has been found that the C-terminal truncated RB was still able to bind cyclin D3 (81). It appears, therefore, that cyclin D3 is also not involved in the process of apoptosis associated with the C-terminal cleavage of RB. MDM2 is a regulatory protein which binds to the C-terminal 137 amino acids of RB (85). The C-terminal cleaved form of RB completely lose its ability to bind to MDM2 (81). The consequence of the lack of MDM2 binding may be the selective inactivation of RB function or the release of free MDM2, which might contribute to the initiation of apoptotic process.

5. P110/UNPHOS/RB AS AN INHIBITOR OF APOPTOSIS

5.1. Inhibitory effects of p110/unphos/RB on apoptosis

Results from Rb gene-knock out experiments suggest that the RB protein may have inhibitory effects on apoptosis (28-30). This hypothesis was also investigated by transfection of wild type Rb gene into RB mutant or null cells. Transfection of Rb gene into those cells resulted in overexpression of RB protein in the p110/unphos form (23). When the RB-defective carcinoma cell lines and their respective RB-reconstituted sister clones were treated with IFN-gamma, only the RB-defective cells, but not the RB-reconstituted clones, formed the apoptosis-associated DNA ladder (86), supporting the hypothesis that p110/unphos/RB has an apoptosis-inhibitory function. Treatment with IFN-gamma induced higher molecular weight DNA in both the RB-defective and the RB-reconstituted cells (86), indicating that the transfection of RB into these cells can only block the IFN-gamma-induced apoptosis at an early stage, but not inhibit apoptosis completely.

Hela cells constitutively expressing HPV proteins contain very little functional p53 and RB activities. Transient overexpression of wild-type p53 in these cells induced apoptosis, while co-expression of functional RB resulted in significant protection of Hela cells from p53-mediated apoptosis (87). These data indicate that RB plays a major role in the decision of whether a cell undergoes growth arrest or apoptosis in response to activation of p53. We have found that in the process of p53-independent apoptosis, while the p120/hyper/RB was converted to p115/hypo/RB and then cleaved, the level of the p110/unphos/RB was unchanged (14), support the idea that the unphosphorylated form of RB is also an inhibitor for p53-independent apoptosis.

Human osteogenic sarcoma cell line SAOS-2 contains a mutant form of RB. When this cell line was treated with ionizing radiation, apoptosis occurred in a time- and dose-dependent manner (88). In both transient and stable transfection assays, SAOS-2 derivatives expressing wild-type RB exhibited increased viability and decreased apoptosis following treatment at a variety of radiation doses. Expression of a mutant RB protein, which has lost the function of forming a complex with the adenovirus E1A oncoprotein or the cellular E2F transcription factor, failed to protect SAOS-2 cells from undergoing apoptosis (88). These results suggest that inhibition of apoptosis by RB is through inhibition of E2F-mediated gene transcription and that E2F has a novel function in promoting apoptosis.

During neuronal apoptosis, cyclin D1-dependent kinase activity is increased, due to an increase in cyclin D1 protein levels (89). Artificial elevation of cyclin D1 levels is sufficient to induce apoptosis in these cells, which is inhibited by expression of the Rb gene (89). This data suggests that p110/unphos/RB is an endogenous target of cyclin D1-dependent kinases during apoptosis. Taken together, these results demonstrate that p110/hypo/RB has an inhibitory function on apoptosis.

5.2. Inactivation of p110/unphos/RB is associated with induction of apoptosis

Since overexpression of p110/unphos/RB in RB deficient cells inhibits apoptosis, one would predict that inactivation of the endogenous p110/unphos/RB should induce apoptotic death process. Indeed, apoptosis is one of the cellular responses to any one of the following processes that could inhibit the activity of p110/unphos/RB.

(i) Deletion of the Rb gene. Experiments using transgenic mice demonstrated that inheritance of two mutant RB alleles ($Rb^{-/-}$) results in lethality at 13-15 days of gestation (28-30). Histological analysis of Rb-deficient embryos revealed that in the peripheral and central nervous systems, widespread apoptotic cell death occurred, as evidenced by nuclear fragmentation, chromatin condensation and TUNEL (TdT-mediated dUTP-biotin nick-end labeling) staining (28-30). When embryos deficient for both of RB and p53 genes ($Rb^{-/-}$ and $p53^{-/-}$) were examined, apoptosis was significantly blocked (90). In another experiment, treatment with anticancer agents accumulated p53 protein in all the mouse embryonic fibroblast cells containing $Rb^{-/-}$, $Rb^{+/-}$ or $Rb^{+/+}$. However, induction of p53 leads to apoptosis in $Rb^{-/-}$ cells, but to growth arrest in both $Rb^{+/-}$ and $Rb^{+/+}$ cells (91). Taken together, these data suggest that induction of apoptosis by Rb gene deletion is p53-dependent.

(ii) Expression of viral oncoproteins**.** It has been reported that only the un- or hypo-phosphorylated form of RB binds to DNA viral proteins, such as HPV16 E7 and adenovirus E1A, resulting in inactivation of RB function (15-17). Rat kidney cells expressing adenovirus E1A remain susceptible to induction of cell death, as evident by intranucleosomal DNA fragmentation and chromatin condensation. Overexpression of Bcl-2 overcomes E1A-induced apoptosis (9). In another experiment, the transgenic mice expressing HPV16 E7 revealed inhibition of cell differentiation and induction of apoptosis (92, 93).

In addition, apoptosis was also induced in normal human fibroblasts expressing HPV16 E7 (94).

(iii) Induction of cyclin-dependent kinases. RB is phosphorylated by several CDK kinases during cell cycle progression (18, 19). If expression of p110/unphos/RB inhibits induction of apoptosis, expression of a CDK kinase component should be associated with induction of apoptosis. Indeed, the levels of cyclin D1 protein and its dependent kinase activity increased during neuronal apoptosis. Furthermore, artificial elevation of cyclin D1 was sufficient to induce apoptosis (89). The cyclin D1-induced apoptosis can be suppressed by overexpression of p16/INK4A, the specific inhibitor of cyclin D-dependent kinases (89), indicating that activation of endogenous cyclin D1-dependent kinases is essential for neuronal apoptosis. Forced expression of p16/INK4A or p21/Waf-1 also inhibited apoptosis during myocyte differentiation (95). It has also been shown that induction of cyclin A associated-kinases was involved in apoptosis induced by c-Myc, Zn^{2+} or granzyme B (96-98).

(iv) Inhibition of RB expression by its antisense oligonucleotides. Transfection of an RB antisense (but not sense) oligonucleotide into cultured primary rat hepatocytes blocked expression of RB, accompanied with induction of apoptosis, as shown by a decrease in the number of viable cells, an increase in nicked DNA, and apoptotic nuclear changes (65).

(v) Expression of E2F transcription factor. Since E2F is one of the RB binding proteins and since overexpression of p110/unphos/RB is associated with inhibition of both cell growth and apoptosis, E2F may function as an activator in processes of both proliferation and cell death. Indeed, it has been reported that deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis (99-102). Coexpression of the wild type of p53 and E2F-1 also resulted in a rapid loss of cell viability through the process of apoptosis, which was inhibitable by expression of a mutant p53 (99-102), indicating that E2F-induced apoptosis is p53-dependent. The E2F-mediated apoptosis can also be inhibited by co-expression of wild-type Rb gene (102).

5.3. Possible molecular mechanisms for p110/unphos/RB as an inhibitor of apoptosis

Mechanisms for the Inhibitory effects of p110/unphos/RB on apoptosis are unclear. Although it has been suggested that inhibition of E2F-mediated gene transcription may be involved, other mechanisms may also be possible such as involvement of blocking the apoptosis-associated RB phosphatase and the RB cleavage enzyme activities. This hypothesis is supported by the following results. First, it has been found that the un-phosphorylated form of RB has the ability to bind to protein phosphatase(s). For example, during the M to G1 transition, the un-phosphorylated form of RB binds to the catalytic subunit of a novel type 1 protein phosphatase (21). It is possible that the overexpressed p110/unphos/RB might interact with the RB phosphatase, blocking its mediated conversion of p120/hyper/RB to p115/hypo/RB, the proper form of RB that can be subsequently cleaved by a caspase. Secondly, we found that a bacteially-expressed or *in vitro* translated RB protein (in the un-phosphorylated form) can also be cleaved when incubated with a cell extract prepared from the drug-pretreated but not control cells (unpublished data). This result suggests that although during cellular apoptosis, p115/hypo/RB, but not p110/unphos/RB, is preferentially cleaved by a caspase protease, p110/unphos/RB at an artificially overexpressed level could compete with the endogenous, limited amount of p115/hypo/RB for the RB cleavage activity.

6. SUMMARY

Investigations in our and other laboratories have demonstrated that RB is involved in the process of apoptosis. Activation of caspases is responsible for the interior and C-terminal cleavage of RB. The cleavage products of RB have an altered affinity to some RB binding proteins. Future studies should focus on further identification of these RB cleavage enzymes and their regulation during apoptosis. These studies will help to define the process of apoptosis at the molecular level and to improve therapies from human diseases caused by dysregulation of apoptosis.

7. ACKNOWLEDGMENTS

Supported in part by a National Institutes of Health Grant AG13300 to QPD.

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Key words: Apoptosis; Caspases, Cell Cycle, Proteolysis, RB

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