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Characterization of Interior Cleavage of Retinoblastoma Protein in Apoptosis

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Abstract Previously we reported that at the onset of apoptotic execution, retinoblastoma protein (RB) was cleaved in its interior region, resulting in production of two major fragments, p48 and p68, and that the RB interior cleavage was mediated by a caspase-like activity. Here, we further characterized the RB interior cleavage process in human leukemia cells treated with the anticancer agent etoposide. We found that the RB interior cleavage activity was much more sensitive to two specific tetrapeptide caspase inhibitors, YVAD-CMK and DEVD-FMK, than the poly(ADP-ribose) polymerase cleavage activity, suggesting that two distinct caspases are involved in these processes. Several Asp residues are located in amino acids 341–421 of RB protein, and cleavage of any one of these sites by a caspase would generate a p48, which contains the amino terminus, and a p68 fragment, which contains the A/B pocket and the carboxyl terminus. This hypothesis was supported by the fact that the p48 and p68 fragments had selective binding affinity to different RB antibodies and that the p48 was found only in the low-salt-extracted cytoplasmic fraction, while the p68 was only in the nuclear fraction, of the apoptotic cells. However, 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 unphosphorylated form of RB, but not the p68 fragment. Lastly, we confirmed that RB also underwent dephosphorylation and carboxyl terminal cleavage during apoptosis, as we and others reported previously. J. Cell. Biochem. 67:399–408, 1997.

Key words: caspase; apoptosis; CPP32; ICE; PARP; RB

Apoptosis, or programmed cell death, is a morphologically and biochemically distinct form of cell death that occurs in most cell types and organisms [Wylie et al., 1980]. Cells achieve their own death through activation of a genetically encoded program that results in cytoskeletal and nuclear disruptions, cell shrinkage, and membrane blebbing [Williams et al., 1993; Steller, 1995; Thompson, 1995]. Investigation into the mechanism of apoptosis in mammals suggests that various stimuli can trigger the apoptotic pathway. It has been shown that proteases of ICE/CED-3 family, recently termed caspases, are intimately involved in the regulation of apoptosis induced by many stimuli. At least ten members of caspase family have been identified, each with a conserved QACRG pentapeptide catalytic site [Duan et al., 1996; Alnemri et al., 1996]. It has also been shown that a number of cellular proteins are cleaved during the onset of apoptosis by the caspases, including poly(ADP-ribose) polymerase (PARP), the 70 kDa component of the U1 small ribonucleoprotein (U1-70 kDa), and lamin A [Patel et al., 1996]. Cleavage of these proteins occurs at a tetrapeptide motif containing an aspartic acid in the P1 position and can be blocked through inhibition of protease activity by tetrapeptide mimics of the substrate cleavage site (i.e., YVAD-CMK, DEVD-FMK), the cowpox virus CrmA protein, or the Bcl-2 oncoprotein [Thompson, 1995; Thornberry et al., 1992].

The RB protein is a tumor suppressor implicated in controlling tumorigenesis in many human cancers. RB is regulated throughout the cell cycle by phosphorylation, being hypophosphorylated at G0/G1, becoming phosphorylated

Abbreviations: CED-3, cell death defective gene 3; CrmA, cytokine response modifier A; DEVD-FMK, acetyl-Asp-Glu-Val-Asp-fluoromethyl-ketone; ICE, interleukin-1β-converting enzyme; PARP, poly(ADP-ribose) polymerase; RB, retinoblastoma protein; U1-70 kDa, the 70 kDa component of the U1 small ribonucleoprotein; VP-16, etoposide; YVAD-CMK, acetyl-Tyr-Val-Ala-Asp-chloromethyl-ketone.

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just prior to the G1/S transition, and subsequently dephosphorylated at the M/G1 transition [Wang et al., 1994; Weinberg, 1995]. The hypophosphorylated but not the hyperphosphorylated form of RB has been suggested as an endogenous inhibitor of cell proliferation [Wang et al., 1994; Weinberg, 1995]. Recently, our laboratory has found that in an early stage of DNA-damage-induced, p53-independent apoptosis, RB is dephosphorylated to a specific p115 form, which is mediated, at least in part, by an activated serine/threonine phosphatase [Dou et al., 1995]. Subsequently, accompanying the internucleosomal fragmentation of DNA, the newly formed p115/hypo/RB is cleaved into at least two fragments, p48 and p68, which is mediated by a protease that has properties of a caspase enzyme [An and Dou, 1996].

In this communication we further investigated the properties of the RB interior cleavage process in human Jurkat T or HL-60 cells treated with the anticancer agent etoposide. We demonstrate that the RB interior cleavage activity possessed greater sensitivity to YVAD-CMK and DEVD-FMK than the PARP cleavage activity. By searching the RB sequence, we identified several Asp residues located between amino acids 341–421. Cleavage of any one of these sites by a caspase would generate a p48 (containing the amino terminus) and a p68 fragment (containing the A/B pocket and the carboxyl terminus). This hypothesis was supported by the fact that the p48 and p68 fragments had selective binding affinity to different RB antibodies and that the p48 was found only in the low-salt-extracted cytoplasmic fraction, while the p68 was only in the nuclear fraction, of the apoptotic cells. Coimmunoprecipitation experiments demonstrate that the nuclear binding partner of the p68/RB fragment is not the transcription factor E2F-1. Lastly, we confirmed that during apoptosis, RB underwent not only interior cleavage, but also dephosphorylation and carboxyl terminal cleavage, as we [Dou et al., 1995] and others [Morana et al., 1996; Wang et al., 1996; Janicke et al., 1996; Chen et al., 1997; Tan et al., 1997] reported previously.

MATERIALS AND METHODS

Materials

Mouse monoclonal culture supernatants to human RB (XZ55, XZ104) and E2F-1 protein were a kind gift from Drs. N. Dyson and E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA). Purified mouse monoclonal antibody to human RB protein (G3-245) was purchased from PharMingen (San Diego, CA); to human PARP (C2-10) was from Unité de Santé et Environnement (Québec, Canada); to E2F-1 and RB (C-15) from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody to E2F-1 was purchased from Upstate Biotechnology (Lake Placid, NY). The specific tetrapeptide inhibitor YVAD-CMK was obtained from Bachem (King of Prussia, PA); DEVD-FMK was from Kamiya Biomedical Company (Seattle, WA). Etoposide (VP-16), tamoxifen, and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell Cultures

Human Jurkat T and HL-60 cells were grown as described previously [An and Dou, 1996; Dou et al., 1997]. Human Jurkat T cells transfected with a plasmid containing either the crmA or the bcl-2 gene were obtained from the laboratory of Dr. Daniel E. Johnson (University of Pittsburgh) and grown as described [Dou et al., 1997].

Whole Cell, Cytoplasmic, and Nuclear Extracts

Whole cell extracts were prepared in lysis buffer as described [An and Dou, 1996]. For cytoplasmic and nuclear extracts, cells were washed once with PBS before being homogenized in EBD buffer [Martin et al., 1995]. After 10 min incubation at 4°C, the mixtures were centrifuged, and the supernatants were collected as cytoplasmic extracts. Remaining pellets were washed twice with EBD buffer and then resuspended in Buffer C [Dignam et al., 1993]. Mixtures were then incubated at 4°C for 30 min, centrifuged, and supernatants were collected as nuclear extracts. Protein concentration of extracts was determined by Bio-Rad (Richmond, CA) assay. Aliquots of extracts were kept at −20°C.

Western Blot Analysis

The enhanced chemiluminescence Western blot assay was performed as described previously [An and Dou, 1996] by using an antibody to RB (XZ55 at 1:4, or G3-245 at 1:200 dilution), PARP (at 1:5,000 dilution), or E2F-1 (polyclonal, at 1:5,000 dilution).

Immunoprecipitation Assay

A whole cell extract (100–200 µg/reaction) was preincubated with 3–5 µg or 20 µl of antibody (XZ104, G3-245, C-15, or E2F-1) at 4°C for 1 h, followed by addition of 10 µl of protein G-PLUS/protein A-agarose in buffer B (50 mM Tris pH 7.5, 0.1% NP-40, 250 mM NaCl, and 5 mM EDTA). After 2 h of vortexing of this mixture, the pellet was washed with buffer B and subjected to Western blot analysis.

RESULTS

Comparison Between the RB Interior Cleavage and the PARP Cleavage

Our previous experiments demonstrated that during the process of DNA-damage-induced apoptosis, RB was cleaved into two major fragments, p48 and p68, detected by specific antibodies G3-245 and XZ55, respectively [An and Dou, 1996]. Since antibody G3-245 recognizes amino acids 300–380 [Ludlow et al., 1989] and antibody XZ55 interacts with amino acids 443–622 of RB [Hu et al., 1991], it appears that the cleavage occurs in the interior region of RB, resulting in production of the RB fragments p48 (from the amino terminus of RB to the cleavage site) and p68 (from the cleavage site to the carboxyl terminus) (Fig. 1).

We also determined that the process of the RB interior cleavage is mediated by a caspase activity since it was inhibitable by three different caspase inhibitors, YVAD-CMK [An and Dou, 1996], Bcl-2, and CrmA [Dou et al., 1997]. Other groups have reported that PARP is cleaved into a p85 and a p25 fragments during apoptosis by a caspase-3/CPP32-like activity [Kaufmann et al., 1993; Tewari et al., 1995]. To further understand the nature of the RB interior cleavage enzyme, we compared the processes of drug-induced RB and PARP cleavage. We first compared sensitivity of these two processes to specific tetrapeptide inhibitors of caspases, YVAD-CMK and DEVD-FMK [Thornberry et al., 1992; Nicholson et al., 1995]. When VP-16-treated Jurkat T cells were incubated with YVAD-CMK, production of the p48/RB fragment was effectively blocked at 1 µM (Fig. 2A, lane 4 vs. 2). In contrast, cleavage of PARP was relatively resistant to addition of YVAD-CMK: no inhibition at up to 1 µM, little inhibition at up to 10 µM, and a complete inhibition only when used at a concentration of 25 µM or higher (Fig. 2B, lanes 3–8 vs. 2). When DEVD-FMK was added to the drug-treated cells, production of the p48/RB fragment was also blocked at 1 µM (Fig. 3A, lane 5 vs. lane 2). However, PARP cleavage was prevented by this inhibitor only at

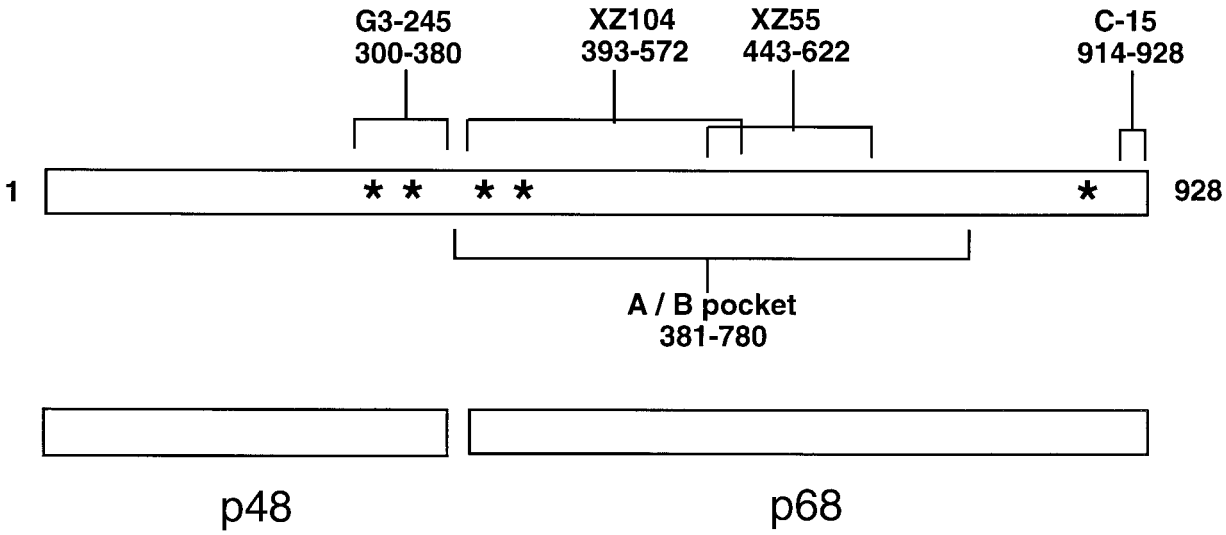

Fig. 1. Candidate caspase cleavage sites in RB. The amino acid specificity of each of the mouse monoclonal antibodies used in this study is shown above: G3-245 recognizes amino acids 300–380; XZ104 recognizes amino acids 393–572; XZ55 recognizes amino acids 443–662; and C-15 recognizes amino acids 914–928. These specific amino acid sequences have allowed us to focus on four Asp residues on amino acids 349, 363, 394, and 421 (indicated by *) of human RB protein that could serve as putative interior cleavage sites. Cleavage at any one of these sites would yield two fragments: a p48 fragment containing the N-terminus of RB and a p68 fragment containing the A/B pocket and C-terminus. Also shown is the most recently characterized C-terminal cleavage site (also indicated by *) at Asp^{886}.

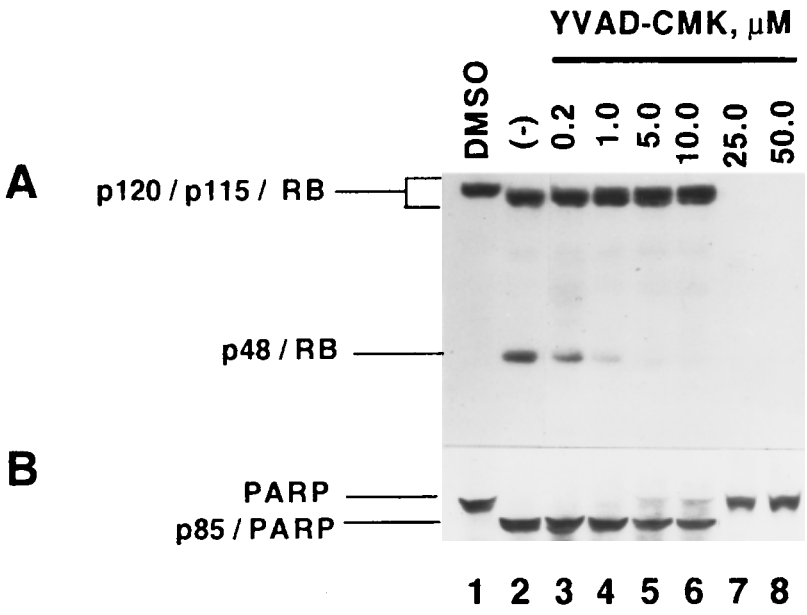

Fig. 2. Inhibition of VP-16-induced RB and PARP cleavage by YVAD-CMK. Jurkat T-lymphocytes were treated with either 50 μM VP-16 (**lanes 2–8**) or an equal percentage (0.03%) DMSO (**lane 1**) for 10 h, in the absence or presence of YVAD-CMK at the indicated concentrations. Aliquots of cellular extracts (50 μg/lane) were examined by Western blot analysis using a monoclonal antibody to human RB (G3-245; **A**) or PARP (**B**). The full-length forms of RB (p120/p115/RB) and PARP as well as their cleavage fragments (p48/RB, p85/PARP) are indicated.

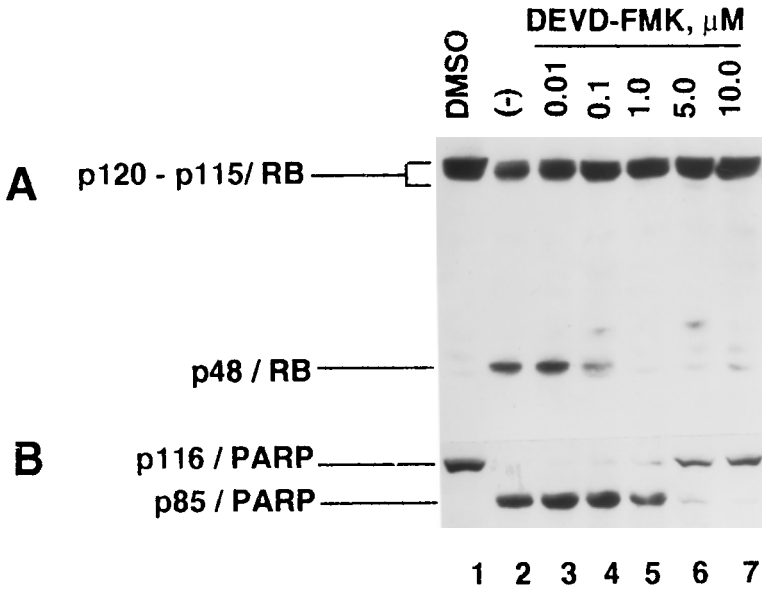

Fig. 3. Inhibition of VP-16-induced RB and PARP cleavage by DEVD-FMK. Jurkat T-lymphocytes were treated with either 50 μM VP-16 (**lanes 2–7**) or DMSO (**lane 1**) for 10 h, in the absence or presence of DEVD-FMK at the indicated concentrations. Aliquots of cellular extracts (50 μg/lane) were examined by Western blot analysis using a monoclonal antibody to human RB (G3-245; **A**) or PARP (**B**), as described in the legend of Figure 2.

5 μM or higher (Fig. 3B, lanes 6 and 7 vs. 2). Therefore, the RB interior cleavage was more sensitive to both tetrapeptide caspase inhibitors than PARP cleavage. It appears that PARP cleavage was more sensitive to DEVD-FMK than to YVAD-CMK (5–10 μM vs. 25 μM; compare Fig. 3B to Fig. 2B). This was consistent with the notion that PARP is cleaved during apoptosis by a caspase-3-like enzyme, which is inhibited directly by DEVD-FMK but not by YVAD-CMK [Nicholson et al., 1995].

In contrast to their different sensitivities to tetrapeptide caspase inhibitors, the processes of RB and PARP cleavage were equally sensitive to two other inhibitors of caspases and apoptosis: the viral protein CrmA [Ray et al., 1992] and the oncoprotein Bcl-2 [White, 1996]. While expression of CrmA delayed by ~2 h VP-16-induced processes of both RB and PARP cleavage, expression of Bcl-2 completely blocked both cleavage processes (data not shown). Taken together, these results suggest that the caspase activity that mediates the RB interior cleavage is similar to, but different from, the PARP cleavage activity.

Specificity of p48/RB and p68/RB Fragments to Different RB Antibodies

Toward the goal of eventually identifying the RB interior cleavage site through which both

p48/RB and p68/RB are produced, we further investigated specificity of p48/RB and p68/RB fragments to different antibodies in an immunoprecipitation-Western blotting assay. Whole cell extracts were prepared from VP-16- or DMSO-treated Jurkat-T cells (Fig. 4A and B, lanes 1, 2), and incubated with RB antibodies G3-245 or XZ104 (recognizing amino acids 393–572 of RB) [Hu et al., 1991]. Aliquots of the immunoprecipitates were analyzed in Western blot assay using RB antibody G3-245 or XZ55.

When the G3-245 immunoprecipitate was blotted with the same antibody, the p48/RB cleavage fragment was detected only in cells treated with VP-16 but not DMSO (Fig. 4A, lane 4 vs. 3). However, when an aliquot of the G3-245 immunoprecipitate was blotted with antibody XZ55, little or no p68/RB fragment was detected (Fig. 4B, lane 4 vs. 2). This result supports the idea that p48, but not p68, fragment of RB contains a sequence of amino acids 300–380. When the XZ104 immunoprecipitate was blotted by antibody XZ55, the p68/RB fragment was detected from cells treated with VP-16 but not DMSO (Fig. 4B, lane 6 vs. 5). However, blotting of the XZ104 immunocomplex with antibody G3-245 detected only intact RB proteins but not p48/RB fragment (Fig. 4A, lanes 5, 6). Therefore, the RB cleavage product p68, but not p48, contains the sequence of amino acids 393–572 (the XZ104-recognized region; see Fig. 1).

These results, together with that from experiments using caspase inhibitors (Figs. 2, 3), strongly suggest that during apoptosis, an activated caspase, which is different from caspase-3, cleaves an interior Asp residue of RB, resulting in production of p48 and p68 fragments. Consistent with this hypothesis, we identified four Asp residues located on amino acids 349, 363, 394, and 421 of human RB protein (Fig. 1). Cleavage of any one of these Asp sites could produce a p48 fragment containing the N-terminal sequence of RB and a p68 fragment containing the A/B pocket (amino acids 381–780) and C-terminal sequence (see Fig. 1).

Cellular Localization of RB Cleavage Fragments

Early immunofluorescence experiments demonstrated the nuclear localization of RB protein [Mittnacht and Weinberg, 1991]. However, when cytoplasmic (by using a low-salt buffer) and nuclear (by a high-salt buffer) extracts were

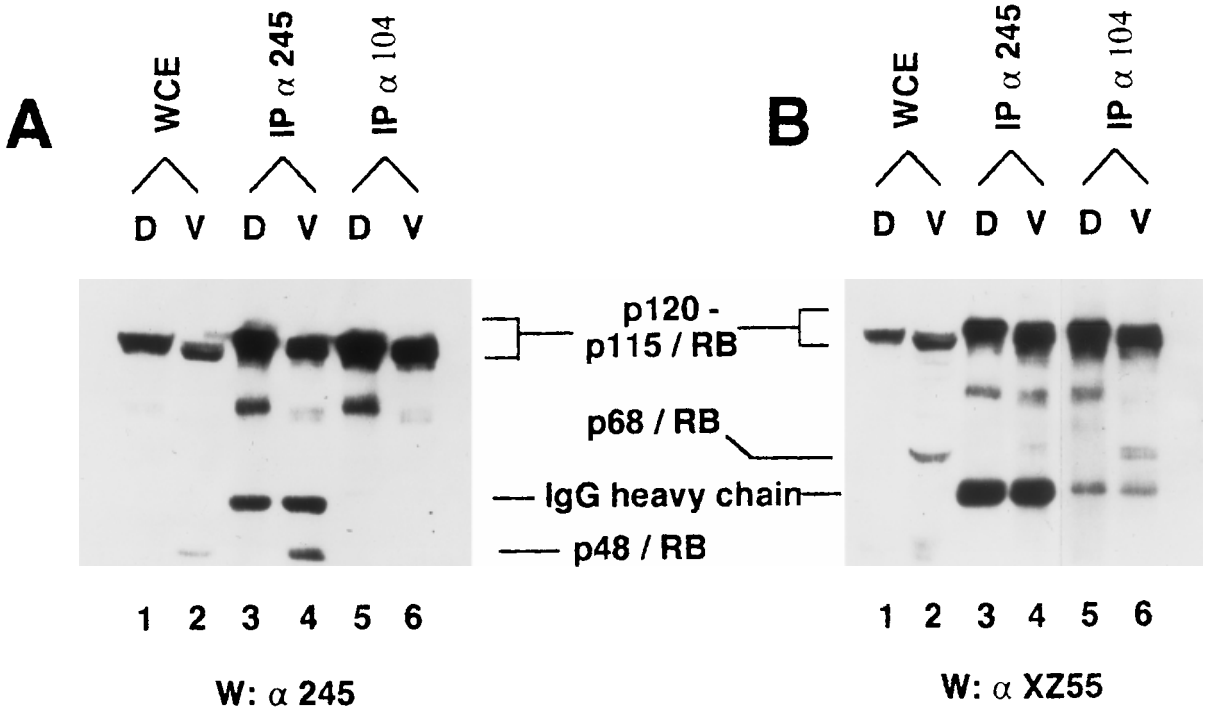

Fig. 4. Specific interactions of RB antibodies and cleavage fragments. HL-60 cells were treated with 30 μM VP-16 for 8 h. Whole cell extracts (200 μg/reaction) were then immunoprecipitated with the RB antibodies G3-245 (A and B, **lanes 3** and **4;** 3 μg/reaction) or XZ104 (A and B, **lanes 5** and **6;** 20 μl/reaction). Aliquots of the immunoprecipitates were analyzed by Western blot using antibody G3-245 (**A**) or XZ55 (**B**). The full-length forms of RB (p120-p115/RB), the p48/RB and p68/RB fragments, and the IgG heavy chain of the immunoprecipitating antibodies are indicated. Western blots of whole cell extracts (A and B, **lanes 1** and **2;** 50 μg/lane) are used as controls.

prepared from cells, the phosphorylated form of RB was found in the cytoplasmic fraction while the unphosphorylated form was in the nuclear fraction [Mittnacht and Weinberg, 1991]. This is most likely because the unphosphorylated, but not the phosphorylated, form of RB interacts with several cellular DNA-binding proteins, i.e., the E2F family of transcription factors [Nevins, 1992; Weinberg, 1996].

We investigated cellular localization of RB cleavage products by analyzing both cytoplasmic and nuclear fractions prepared from VP-16-treated Jurkat cells (see Materials and Methods). Western blotting with G3-245 detected the p48/RB fragment in the cytoplasmic but not the nuclear fraction of the VP-16-treated cells (Fig. 5A, lanes 1–3 vs. 4–6). However, the majority of the p68/RB fragment was detected in the nuclear fraction of the same cells by antibody XZ55 (Fig. 5B, lanes 4–6 vs. 1–3). The decrease in the level of p68/RB at a later time point was probably due to additional proteolysis of this fragment (Fig. 5B, lane 6 vs. 5). The cytoplasmic localization of p48/RB and the nuclear localization of p68/RB were also found in human breast cancer MDA-MB-231 cells that had been treated with VP-16 or tamoxifen (data not shown). Therefore, the p68/RB, but not p48/RB, fragment may retain the ability to interact with some nuclear components. This was consistent with the hypothesis that the p68 but not the p48 fragment contains the "A/B" pocket and C-terminal sequence (see Fig. 1), which are required for interaction of RB with some cellular nuclear proteins [Qin et al., 1992; Kaelin et al., 1991]. We also found most of the p85/PARP cleavage product in the nuclear fraction (Fig. 5C, lanes 4–6 vs. 1–3), indicating that this cleavage product may still retain DNA binding activity.

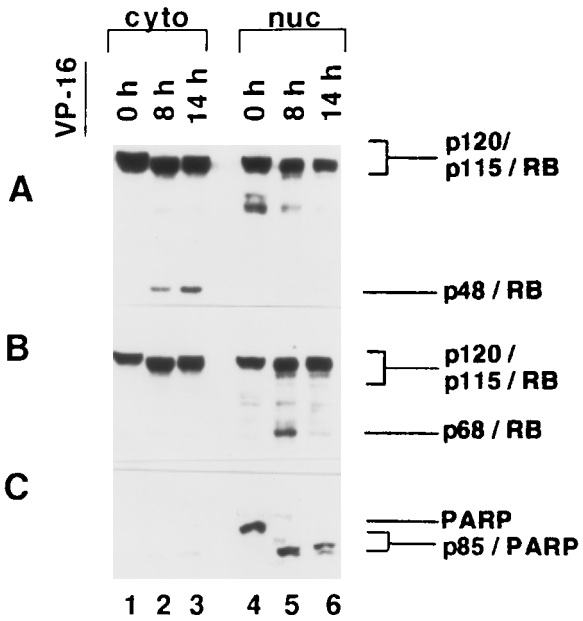

Fig. 5. Cellular localization of cleavage products of RB and PARP. Human Jurkat T-lymphocytes were treated with 50 μM VP-16 for the indicated hours, followed by preparation of both cytoplasmic (cyto) and nuclear (nuc) fractions. Aliquots of these fractions (50 μg/lane) were analyzed in Western blotting using an antibody to human RB (G3-245 in **A,** or XZ55 in **B**), or PARP (C2-10 in **C**), as described in the legend of Figure 2.

p68/RB Cleavage Product Does Not Interact With E2F-1

Since the data presented above suggested that the p68/RB cleavage fragment may have the ability to interact with nuclear components, we investigated the possibility whether this fragment could interact with E2F-1, an RB-binding nuclear transcription factor [Nevins, 1992; Weinberg, 1996]. Whole cell extracts of DMSO- or VP-16-treated HL-60 cells were subjected to immunoprecipitation with a monoclonal antibody to E2F-1, and aliquots of the prepared E2F-1 immunocomplexes were then analyzed by Western blot assay using antibodies to E2F-1 (as a control) or RB. Equal levels of E2F-1 protein were detected in the E2F-1 immunoprecipitates prepared from both DMSO- and VP-16-treated cells (Fig. 6A, lanes 1, 2). When an aliquot of these E2F-1 immunoprecipitates was blotted with the RB monoclonal antibody XZ-55, the p110/unphosphorylated form of the RB protein was most prevalent (Fig. 6B, lanes 1, 2). However, the p68/RB fragment was not found in the preparation from VP-16-treated cells (Fig. 6B, lane 2 vs. lane C, the control whole cell extract; also compare to Fig. 4B, lane 6). We also repeated this experiment with several different E2F-1 antibodies and found that none of them could co-immunoprecipitate the p68/RB fragment (data not shown). Therefore, we conclude that although p68/RB is located in the nucleus (Fig. 5B), its binding partner is not E2F-1.

RB Dephosphorylation and C-Terminal Cleavage During Apoptosis

Previously, we [Dou et al., 1995] and others [Morana et al., 1996, Wang et al., 1996] reported dephosphorylation of RB prior to the initiation of apoptotic execution. Very recently,

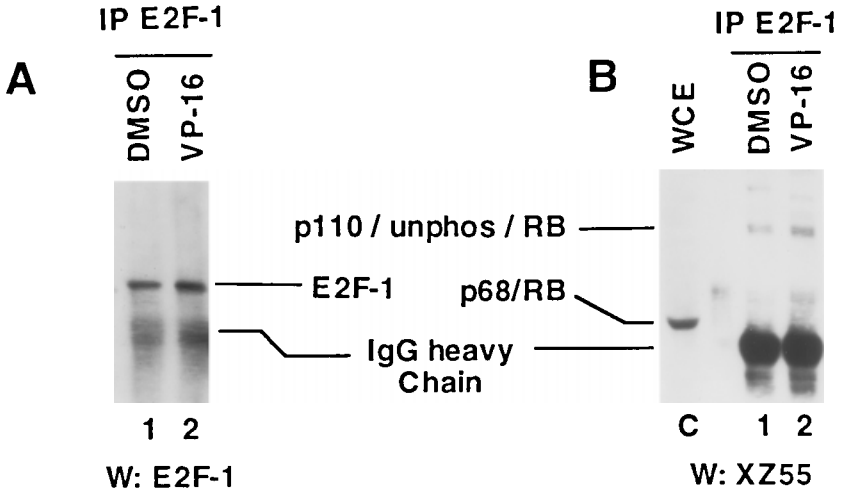

Fig. 6. The E2F-1 antibody does not coimmunoprecipitate the p68/RB fragment. HL-60 cells were treated with 30 μM VP-16 for 8 h. Whole cell extracts (200 μg/reaction) were immunoprecipitated with a mouse monoclonal antibody to E2F-1 (20 μl/reaction). Aliquots of the immunoprecipitates were analyzed by Western blot assay using a rabbit polyclonal E2F-1 antibody (**A**) or the mouse monoclonal RB antibody XZ55 (**B**). Lane C in B is a control whole cell extract (WCE) that contains the p68/RB fragment. The E2F-1 protein, the full-length unphosphorylated form of RB (p110/unphos/RB), the p68/RB fragment, and the IgG heavy chain are indicated.

several groups reported that during apoptosis the C-terminal 42 amino acid of RB is cleaved off by a caspase-3-like enzyme [Chen et al., 1997; Janicke et al., 1996; Tan et al., 1997], and the process of the C-terminal cleavage might mimic dephosphorylation of RB [Chen et al., 1997]. To investigate whether both RB dephosphorylation and C-terminal cleavage occur during apoptosis, we prepared RB immunoprecipitates using antibodies G3-245 and C-15, and analyzed these immunocomplexes in Western blot assay using G3-245 antibody. The C-15 antibody recognizes the C-terminal 15 amino acids of RB (Fig. 1) and, therefore, should not interact with the C-terminal truncated form of RB.

When the G3-245 immunoprecipitate, prepared from the whole cell extract of the control HL-60 cells, was analyzed, only the hyperphosphorylated form of RB, p120/hyper, was detected (Fig. 7A and B, lane 1). However, when a preparation from cells treated with VP-16 for 4 h was analyzed, the level of immunoprecipitated p120/hyper was dramatically decreased, associated with the appearance of an abundant band (p115/hypo/RB) and a weak band (112/clv/RB) of RB (Fig. 7, lane 2 vs. 1). At 8 h of treatment, the levels of both p115/hypo/RB and p112/clv/RB forms of RB decreased (Fig. 7A and B).

The C-15 antibody was able to immunoprecipitate the p120/hyper form of RB from the control cells (Fig. 7A and B, lane 4), whose level was comparable to that immunoprecipitated by G3-245 antibody (compare to lane 1), suggesting that both antibodies have similar affinity to the hyperphosphorylated RB. When VP-16-treated cells were used, the C-15 antibody only precipitated a low level of p115/hypo/RB (Fig. 7, compare lanes 5, 6, vs. 2, 3). This data suggest that the C-15 antibody has weaker affinity to p115/hypo/RB than the G3-245 antibody. The C15 antibody did not immunoprecipitate the p112/clv/RB (lanes 5, 6, vs. 2, 3), confirming that it is C-terminal-truncated. Therefore, during apoptosis RB undergoes not only interior cleavage, but also dephosphorylation and C-terminal cleavage.

DISCUSSION

In this communication, we further examined the process of RB interior cleavage during apoptosis. We found both the RB interior cleavage and PARP cleavage were inhibitable by four caspase inhibitors: YVAD-CMK (Fig. 2), DEVD-FMK (Fig. 3), CrmA (data not shown), and Bcl-2 (data not shown). These results indicate that both proteolytic cleavages are mediated by members of the caspase family. However, the following evidence suggests that two different caspases are responsible for the RB interior and PARP cleavage. First, the RB interior cleavage was blocked by YVAD-CMK and DEVD-FMK at a concentration of 1 μM (Figs. 2A and 3A), while PARP cleavage was blocked at a tenfold or higher concentration (Figs. 2B and 3B), indicating that these two protease activities possess different sensitivities to the tetrapeptide inhibitors. In addition, kinetically, RB cleavage occurred ~1 h after PARP cleavage in the VP-16-treated Jurkat T cells (data not shown), indicating that these two proteases are activated at different times by the drug. Taken together, these studies indicate that two closely related, but distinct, caspase activities mediate

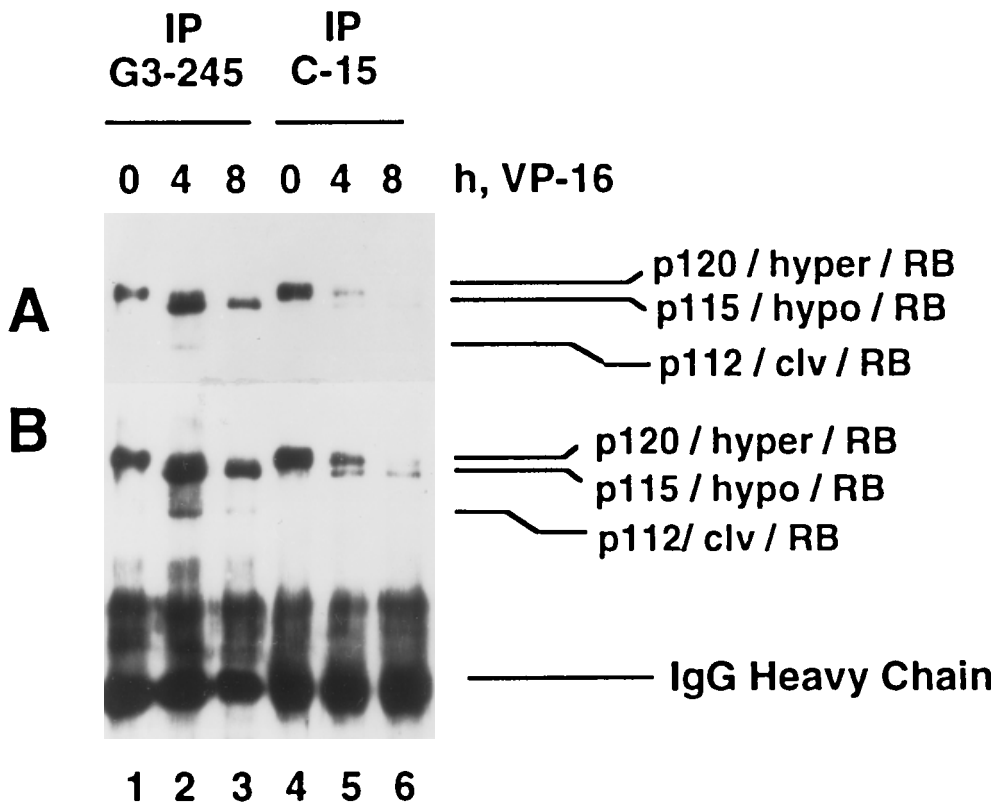

Fig. 7. RB dephosphorylation and C-terminal cleavage during apoptosis. HL-60 cells were treated with 50 μM VP-16 for the indicated hours. Whole cell extracts (100 μg/reaction) were then immunoprecipitated with 5 μg G3-245 (**lanes 1–3**) or C-15 (**lanes 4–6**) RB antibodies. The prepared immunoprecipitates were then analyzed by Western blot assay using antibody G3-245. **A,B:** Short and long exposures of the same ECL membrane. The hyperphosphorylated (p120/hyper/RB), hypophosphorylated (p115/hypo/RB), and the C-terminal cleaved (p112/clv/RB) forms of RB as well as the IgG heavy chain of the immunoprecipitating antibodies are indicated.

the RB interior cleavage and PARP cleavage during apoptosis.

Our studies also indicate that the p68 fragment consists of an RB sequence between the cleavage site and the carboxyl terminus. First, the p68/RB fragment was found in the nuclear fraction of the apoptotic cells (Fig. 5), suggesting that it retains the ability to interact with some cellular DNA-binding protein(s). Secondly, the p68/RB fragment can be detected by antibodies XZ55, XZ77, XZ91, and XZ104 (Fig. 4B and data not shown), all of which recognizes epitopes located in the RB pocket [Hu et al., 1991], but not by antibody G3-245 (Fig. 4A) which reacts specifically with an epitope between amino acids 300–380 [Ludlow et al., 1989]. Knowing that each of these RB antibodies recognize a specific amino acid sequence, we have identified several Asp residues that could serve as candidate cleavage sites for production of both the p48 and p68 RB fragments (Fig. 1). However, more sequencing data is necessary before drawing any definite conclusions.

Previous studies have demonstrated that the RB pocket region is required for interaction with viral oncoproteins and cellular transcription factors and also for mediating RB functions during cell cycle progression and tumor formation [Wang et al., 1994; Weinberg, 1995]. The transcription factor E2F-1 is one of the best characterized proteins known to interact with the A/B pocket of RB [Nevins, 1992]. We found the p68/RB fragment in the nuclear fraction of the apoptotic cells (Fig. 5) and, therefore, wished to investigate the possibility of E2F-1 and p68/RB interaction. Although E2F-1 still interacted with the p110/unphosphorylated form of RB in the apoptotic cells, it did not interact with the p68/RB fragment (Fig. 6). Therefore, the nuclear binding partner of the p68/RB is not the E2F-1 protein. We also found that E2F-1 did not interact strongly with p115/hypo/RB

(Fig. 6), supporting the idea that the p68/RB is generated from the p115/hypo/RB [An and Dou, 1996] but not from the p110/unphos/RB. The E2F-1 antibody did not co-immunoprecipitate the C-terminal truncated form of RB, either (compare Figs. 6, 7), which was in contrast with results from other groups [Chen et al., 1997; Janicke et al., 1996].

Most recently, several groups have demonstrated that RB is also cleaved at a caspase-3 consensus site located in its C-terminus [Janicke et al., 1996; Chen et al., 1997; Tan et al., 1997]. One of these groups suggested that cleavage at this site could "mimic" RB dephosphorylation process but that RB is not actually dephosphorylated during apoptosis [Chen et al., 1997]. Our results presented in this paper are consistent with previous reports from us [Dou et al., 1995] and others [Morana et al., 1996; Wang et al., 1996] that RB indeed undergoes dephosphorylation in apoptosis. We show here that the N-terminal antibody, G3-245, interacted specifically with three forms of RB in cell lysates: the p120/hyper/RB, p115/hypo/RB, and a C-terminal truncated form p112/clv/RB (Fig. 7, lanes 1–3). However, the C-15 antibody was not able to interact with the p112/clv/RB but interacted, although weakly, with the p115/hypo/RB (Fig. 7, lanes 4–6). Hence, it is possible that in the previous studies [Chen et al., 1997] the p115/hypo/RB was overlooked due to the low affinity of the C-15 antibody for this form of RB. The results of our study therefore support the idea that both RB dephosphorylation and C-terminal cleavage occur during apoptosis.

It has been recently shown that as many as nine out of the ten known caspases are activated during etoposide-induced apoptosis in HL-60 cells [Martins et al., 1997]. Furthermore, it is evident now that the caspase family of proteases have both overlapping substrate specificity and at least partially overlapping functions. For example, both caspase-3 and caspase-7 have the ability to cleave PARP [Tewari et al., 1995; Martins et al., 1997]. The equal sensitivity of the RB interior cleavage activity to the tetrapeptide inhibitors YVAD-CMK and DEVD-FMK (Figs. 2 and 3) suggests that several caspases may be able to cleave RB.

Dephosphorylation of RB to the p115/hypo form occurs at an early apoptotic stage [Dou et al., 1995; Morana et al., 1996; Wang et al., 1996], suggesting that this process is probably involved in cell cycle exit and apoptotic commitment. The interior cleavage of RB is tightly associated with the initiation of apoptotic execution [An and Dou, 1996; Dou, 1997] and failure to cleave RB is associated with drug resistance in HL-60 cells [An et al., 1996]. In contrast, the unphosphorylated form of RB may function as an inhibitor of apoptosis [Dou, 1997]. Therefore, a balance of various phosphorylated forms of RB exists in a cell and tipping the balance by either dephosphorylation or cleavage may affect the life-and-death decisions. A further detailed study of the role of RB dephosphorylation and cleavage during apoptosis will help define the process of cell death at the molecular level.

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