Degradation of Retinoblastoma Protein in Tumor Necrosis Factor- and CD95-induced Cell Death*

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Xuqiu Tan‡§, Seamus J. Martin¶
Douglas R. Green®, and Jean Y. J. Wang**

From the ‡Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92037-0347 and the ¶Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121

The product of the retinoblastoma susceptibility gene, RB, is a negative regulator of cell proliferation. Inactivation of RB does not interfere with embryonic growth or differentiation. However, RB-deficient embryos show abnormal degeneration of neurons and lens fiber cells through apoptosis, suggesting that RB may protect against programmed cell death. Consistent with this notion, RB is found to be degraded in tumor necrosis factor (TNF)- and CD95-induced death. A consensus caspase cleavage site at the C terminus of RB is cleaved in vitro and in vivo by proteases related to CPP32 (caspase 3). Mutation of the consensus cleavage site generates a caspase-resistant RB which is not degraded during cell death. Expression of this non-degradable RB is found to antagonize the cytotoxic effects of TNF in Rb-/- 3T3 cells, but this mutant RB cannot attenuate the rapid death induced by anti-CD95 in Jurkat/T cells. These results show that RB is a target of the caspase family of proteases during cell death and suggest that the failure to degrade RB can attenuate the death response toward some but not all death inducers.

Higher eukaryotic cells have the capability to undergo active cell death, and the programmed death process plays an important role in the development as well as the homeostasis of multicellular organisms. Execution of the death program, i.e. apoptosis, requires a number of cysteine proteases (caspases), exemplified by the ced-3 death effector gene product of Caenorhabditis elegans (1), and includes the vertebrate CED-3 homologs that comprise the interleukin-1-converting enzyme (ICE) family of proteases (2). Inhibitors of the caspases, e.g. the cowpox virus CrmA protein, can protect cells from apoptosis (2–5). A number of cellular proteins, both nuclear and cytoplasmic, have been shown to be cleaved by the caspases during apoptosis (6–8).

The retinoblastoma (RB) protein is a negative regulator of cell proliferation (9). Germ line heterozygous mutations of Rb predispose to the development of retinoblastoma in humans and pituitary tumors in mice (10–13). Homozygous mutation of Rb results in embryonic lethality at day 12–15 of gestation, accompanied by the abnormal degeneration of neurons, photoreceptor cells, and the ocular lens fiber cells (11–14). Inactivation of RB function, through the transgenic expression of the human papilloma virus E7 oncoprotein in the retina or the lens, leads to a similar death phenotype (14). These observations suggest that RB, in addition to its function in growth suppression, may also play a role in the suppression of the death program.

The caspases cleave polypeptides between an aspartic acid and a glycine in the consensus sequence DEXDG (6, 15). A caspase consensus cleavage site, DEADDG, is found in the human RB sequence at amino acids 883 to 887, and this site is conserved in the mouse, chicken, and the Xenopus RB. We show in this report that RB is indeed cleaved by a caspase at this consensus site during the death response triggered by the ligation of CD95. Cleavage of RB is also observed in mouse 3T3 cells in TNF-induced death. Mutation at the consensus site blocked RB degradation in vitro and in vivo, and expression of this cleavage-resistant RB is found to antagonize the TNF cytotoxicity. Our findings show that RB is a target of the death effector proteases and suggest that degradation of RB may be required for cells to respond to TNF as a death signal.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody to human CD95 was obtained from Kamiya Biomedical Co. Recombinant tumor necrosis factor α (TNF-α) was obtained from Calbiochem-Novabiochem International. Anti-RB polyclonal antibody 851 was described previously (16). GST and GST-CrmA were purified from Escherichia coli. DEVD-CHO, YVAD-CHO, and Z-VAD-emk peptides were purchased from BACHEM Bioscience Inc. All other chemicals were obtained from Sigma.

RB Mutants and Plasmids—RB-MI and RB-ΔI were prepared by a polymerase chain reaction-based strategy. The nucleotide sequence of each polymerase chain reaction-generated construct was determined in its entirety to verify the mutations. In RB-MI, amino acids Asp-886 and Gly-887 in the ICE/CED-3 cleavage site (DEADG) were substituted by Ala and Glu, respectively. In RB-ΔI, amino acids from Gly-887 to the end were deleted, mimicking the caspase-cleaved product. Wild type RB and the two mutants, MI and ΔI, were cloned into the pcMV vector (17) to make pCMV-RB, pCMV-RB-MI, and pCMV-RB-ΔI. Wild type RB and the two mutants, MI and ΔI, were also cloned into the pEBB vector (18) to make pEBB-RB-WT, pEBB-RB-MI, and pEBB-RB-ΔI.

Cell Lines and Transfections—Cell lines used in this study were cultured under standard conditions. Jurkat/T cells were co-transfected with pBABE-puro (19) plus pEBB, pEBB-RB-WT, pEBB-RB-MI, or pEBB-RB-ΔI by electroporation. Polyclonal populations of transfected cells were selected by obtaining samples with 0.5 μg/ml puromycin for 2 days. Rb-/- 3T3 cells were co-transfected with pCMV-β-galactosidase and pCMV-RB-WT, pCMV-RB-MI, or pCMV-RB-ΔI by the LipofectAMINE method (Life Technologies, Inc.).

Induction of Cell Death—Human Jurkat and CEM cells (1 × 10^6 cells/ml) were treated with 50 ng/ml anti-CD95 antibody, respectively, the cytokotoxic lymphocyte; GST, glutathione S-transferase; Z-VAD-emk, Val-Ala-Asp-chloromethyl ketone; DEVD-CHO, Ac-Asp-Glu-Val-Asp-阿德-海德; YVAD-CHO, Ac-Tyr-Val-Ala-Asp-阿德-海德; TPA, 12-O-tetradecanoylphorbol-13-acetate; PHA, phytohemagglutinin.

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and collected at the indicated times. To inhibit apoptosis, Jurkat cells were preincubated with 10 μM Z-VAD-cmk for 1 h or with 50 ng/ml TPA plus 1 μg/ml PHA for 38 h and then treated with anti-CD95 antibody. At 30 h post-transfection, RB−/−3T3 cells were seeded at a density of 1 × 10⁶ cells per well of a six-well plate and treated with various amounts of TNF-α plus 100 ng/ml actinomycin D for 14 h or treated with various amounts of TNF-α alone for 45 h.

Cell Death Assays—For Jurkat and Jurkat/T cells, dead cells were assessed by staining with 4 μg/ml acridine orange. Apoptotic nuclei stained with acridine orange showed margination and condensation of the dye. Apoptosis was also verified by DNA degradation and the generation of nucleosomal ladders. Live cells were counted by the exclusion of trypan blue. To determine the death of transfected RB−/−3T3 cells, the β-galactosidase activity in the attached live cells and the floating dead cells was measured. The percent β-galactosidase activity in the floating cells relative to the total activity was given as the percent dead cells. Apoptosis of the floating cells was verified by staining with Hoechst dye to reveal the formation of pyknotic nuclei.

In Vitro Cleavage of RB—Reaction mixtures containing CL granules with CEM cell extracts (20 μg) or Saos-2 extracts (30 μg) expressing either RB, RB-MI, or RB-ΔI were incubated at 37°C for 90 min to 3 h. Inhibitors used were GST (10 μM), GST-CrmA (0.0003–10 μM), DEVD-CHO (0.0001–1 μM), or YVAD-CHO (1 μM). CEM and Saos-2 cell-free extracts were prepared as described previously (20, 21).

RESULTS AND DISCUSSION

The human T cell lines, Jurkat and CEM, express CD95 (also known as Fas) and can be killed rapidly by treatment with anti-CD95 (22, 23). CD95-induced cell death is blocked by CrmA, suggesting that the activation of the caspase family of proteases is essential in the apoptotic response (4, 5). Jurkat cells contained a high level of phosphorylated RB (ppRB), which was degraded within 2 h of treatment with anti-CD95 (Fig. 1a). In Jurkat cells, the ppRB bands were first converted to a series of lower bands (lanes 3 and 4 in Fig. 1a) and then to a predominant ΔRB band (lane 7) which is about 5 kDa smaller than unphosphorylated full length pRB (compare lanes 9 and 10). The total amount of RB also decreased, indicating that ΔRB was further degraded. Treatment of CEM with anti-CD95 also induced apoptosis and the rapid degradation of RB (not shown). In both cell types, ΔRB was a detectable intermediate in the degradation process. RB degradation did not reflect a general loss of total cellular proteins, as exemplified by the constant levels of cyclin A throughout the course of cell killing (Fig. 1a).

CD95-induced apoptosis was prevented when Jurkat cells were pre-treated with TPA/PHA or with Val-Ala-Asp-chloromethyl ketone (Z-VAD-cmk), an inhibitor of caspases. Addition of Z-VAD-cmk completely blocked RB degradation (Fig. 1b, lanes 3, 5, and 7) and prevented cell death. TPA/PHA treatment induced a partial dephosphorylation of RB (even lanes in Fig. 1c); further treatment of these cells with anti-CD95 did not cause death, and no ΔRB was detected in the experimental time course (compare odd and even lanes in Fig. 1c). These results show a tight correlation of RB degradation and cell death.

The ability of caspases to cleave RB was further examined in a cell-free system of granzyme B-induced apoptosis. Granzyme B, a serine protease derived from the granules of cytotoxic lymphocytes (CL), has been shown to activate at least one B, a serine protease derived from the granules of cytotoxic cell-free system of granzyme B-induced apoptosis. Granzyme CrmA (lane 6), which blocks the proteolytic activity of granzyme B (25), and by the peptide Ac-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) (lane 6), which does not inhibit granzyme B but does block CPP32 (caspase 3) and related proteases (25). In contrast, Ac-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO), which is more specific for proteases in the ICE (caspase 1) subfamily, did not inhibit RB cleavage (lane 7). The effects of CrmA and DEVD-CHO on RB cleavage were dependent on the concentrations of these inhibitors (Fig. 1b). These results suggested that RB cleavage may be mediated by CPP32 (caspase 3) or a CPP32 (caspase 3) subfamily protease in this system.

A consensus caspase family cleavage site, Asp⁸⁸³-Glu-Ala-Gly⁸⁸⁷ (DEADG), is found in RB and is situated about 5 kDa from the C terminus of RB (Fig. 2d). To determine if RB was indeed cleaved at this site during apoptosis, we constructed two mutants: RB-MI, with substitutions of Asp886 and Gly887 to Ala and Glu, respectively, and RB-ΔI with a truncation at the putative cleavage site (Fig. 2d). The RB-MI mutant lacking the consensus site was completely resistant to cleavage by granzyme B-activated proteases (Fig. 2e, lanes 5–7). As expected, the in vitro cleaved RB migrated identically to the RB-ΔI mutant (Fig. 2e). To determine if RB-MI was also resistant to cleavage in vitro, it was expressed in a line of Jurkat cells that also expressed the SV40 T-antigen (Jurkat/T cells, Fig. 2e). Expression of exogenous RB could not be achieved in Jurkat cells but was possible with the Jurkat/T cells, most likely because T-antigen can inactivate the growth suppression function of RB. Overproduction of RB-WT or RB-MI was induced by the increased levels of ppRB (Fig. 2e, compare lanes 3 and 1 to 2). In cells transfected with RB-ΔI, several new bands, most...
likely corresponding to phosphorylated RB-ΔI (ppRBΔI), were detected (lane 4). When treated with anti-CD95, the exogenous RB-WT was cleaved to ΔRB indicated by the increased intensity of this band (lane 6), and the exogenous RB-ΔI bands were converted to a tight ΔRB consistent with dephosphorylation (lane 8). Interestingly, in cells expressing RB-MI, no increase in the ΔRB band was observed, instead, anti-CD95 treatment led to the partial dephosphorylation of RB-MI and the generation of pRB (lane 7). Taken together, these results demonstrate that RB is indeed cleaved at the consensus site by caspase family proteases in vitro as well as in anti-CD95-treated cells.

Expression of RB-MI did not have a detectable effect on the anti-CD95-induced death in Jurkat/T cells (Fig. 2e, percent apoptotic cells was identical in all samples). To further examine the effect of RB-MI on the death response, we chose TNF-α as the death inducer because the cytotoxic activity of TNF is dependent on the activation of caspases (26). With normal 3T3 cells, TNF-α treatment alone induced an inefficient and protracted death which could be enhanced by actinomycin D (27). A Rb<sup>−/−</sup> 3T3 line, however, underwent efficient apoptosis by treatment with TNF-α alone, although actinomycin D also accelerated the death response (Fig. 3). In separate experiments, we have found that RB, RB-MI, and RB-ΔI all have growth suppression function. In Rb<sup>−/−</sup> 3T3 cells, reintroduction of RB induced G1 increase but not a cell cycle arrest. The RB-WT, RB-MI, or RB-ΔI was each expressed in Rb<sup>−/−</sup> cells, and the RB level was determined after treatment with TNF alone (Fig. 3b) or TNF plus actinomycin D (Fig. 3d). TNF treatment (200 ng/ml, 45 h) led to a decrease of RB-WT and RB-ΔI (Fig. 3b, compare lanes 3 and 4 or lanes 7 and 8), but did not reduce the level of RB-MI (compare lanes 5 and 6). The ΔRB band was not prominent possibly because the further degradation of ΔRB was more efficient in these Rb<sup>−/−</sup> 3T3 cells than it was in Jurkat cells. When these cells were treated with TNF (3 ng/ml) plus actinomycin D for 14 h (Fig. 3d), ΔRB was detected in cells expressing RB-WT (compare lane 6 to lane 2), the level of RB-ΔI decreased (compare lane 8 to lane 4), but the level of RB-MI was found to increase (compare lane 7 to lane 3) suggesting that cells expressing RB-MI might be selectively preserved. The cleavage-resistant RB-MI was stable at all TNF concentrations tested (Fig. 3 and data not shown). This finding suggests that cleavage at the caspase consensus site is a prerequisite for the further degradation of RB in TNF-treated cells.

Cells expressing RB-WT or RB-ΔI were found to be less sensitive to TNF than the parental RB-deficient cells (Fig. 3, a and c). In contrast, cells expressing RB-MI were completely resistant to TNF-α at concentrations that killed cells expressing RB-WT or RB-ΔI (Fig. 3a). Even at a higher concentration of TNF (200 ng/ml), when 50% of the RB-deficient cells and 25% of the RB-WT or RB-ΔI expressing cells were killed, only 5% of the RB-MI cells were dead (Fig. 3a). Thus, the degradation of RB, induced by cleavage at the caspase consensus site, appears to be required for TNF to induce death. The death protection function of RB-MI is not absolute. When death was accelerated by treatment with TNF plus actinomycin D, RB-MI expressing cells were still more resistant than RB-WT cells, but they were killed at a TNF concentration of 30 ng/ml (Fig. 3c). Moreover, the dead cells were found to contain undegraded RB-MI (not shown). This result suggests that RB cannot inhibit the death process per se; however, the preservation of RB can attenuate the death response to TNF.

In addition to TNF and CD95, treatment with cisplatin and the withdrawal of survival factors also induce the cleavage of RB at the C-terminal caspase consensus site. An and Dou (28) have described the degradation of RB to fragments of 68 kDa and 48 kDa in cancer cells exposed to chemotherapeutic agents. In our hands, smaller fragments of RB could also be detected in anti-CD95 and TNF-treated cells but the sizes were variable (not shown). These observations suggest that RB is first

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cleaved at the C-terminal caspase site, and the cleaved product is then further degraded by other types of proteases.

The induced degradation of RB during apoptosis supports the notion that RB plays an active role in antagonizing the death response. Removal of RB is expected to release E2F-1, which can promote S phase entry and induce apoptosis when activated early by TNF-α signal, can cleave at the C-terminal caspase site, and the cleaved product of RB-WT, RB-MI, or RB-ΔI plasmids expressing RB-WT, RB-MI, or RB-ΔI 3T3 cells were co-transfected with plasmids expressing β-galactosidase. The cells were treated with indicated concentrations of TNF-α for 45 h. The percentage of dead cells was determined as described under “Experimental Procedures.” The means and standard deviation of two independent experiments are shown.

FIG. 3. Inhibitory effect of RB-MI on TNF-α-induced cell death. a, RB-MI promotes cell survival. Rb−/− 3T3 cells were treated or untreated with 200 ng/ml TNF-α for 45 h. Both the attached live cells and the floating dead cells were collected, and one-tenth of the culture was loaded in each lane. b, anti-RB immunoblotting of lysates from transfected Rb−/− 3T3 cells treated or untreated with 3 ng/ml TNF-α plus 100 ng/ml actinomycin D for 14 h. The percentage of dead cells was determined as described under “Experimental Procedures.” The means and standard deviation of two independent experiments are shown. c, anti-RB immunoblotting of lysates from transfected Rb−/− 3T3 cells treated or untreated with 3 ng/ml TNF-α plus 100 ng/ml actinomycin D for 14 h. Again, both the attached live cells and the floating dead cells were collected, and one-tenth of the culture was loaded in each lane.

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