RESEARCH COMMUNICATION

Activation of CPP32 and Mch3 α in wild-type p53-induced apoptosis

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DNA-damaging agents induce apoptosis primarily by a p53-dependent pathway. LTR6 cells containing a temperature-sensitive p53 were used to dissect further the mechanisms of p53-induced apoptosis. Apoptosis was accompanied by the processing

and activation of CPP32 and Mch3 α , together with the cleavage of poly(ADP-ribose) polymerase and lamin B₁. These results demonstrate a critical role for the activation of interleukin-1 β -converting enzyme-like proteases in p53-induced apoptosis.

INTRODUCTION

Apoptosis is the morphological description of an active form of cell death, largely distinct from necrosis, that is widespread in multicellular-organism development and plays an important role in tissue homeostasis [1]. Induced by a variety of cellular stresses, apoptosis is highly conserved between species and is characterized, among other features, by profound chromatin condensation and internucleosomal cleavage of DNA [1]. Recently, the importance of a novel family of cysteine proteases has become evident in apoptosis. Studies of the nematode worm *Caenorhabditis elegans* have identified two genes, *Ced3* and *Ced4*, which are absolutely required for cell death [2]. *Ced3* has significant structural and functional homology with interleukin-1 β -converting enzyme (ICE) [3], the founder member of an emerging family of mammalian cysteine proteases which includes CPP32/apopain [4,5], Mch2 [6], NEDD2/Ich1 [7,8], Ich2/TX [9,10], Mch3/ICE-LAP3 [11,12], ICE-LAP6/Mch6 [13,14], MACH/FLICE/Mch5 [15–17] and Mch4 [17]. These cysteine proteases exist primarily as inactive proforms in normal cells and require processing at critical aspartate residues for activation (reviewed in [18]). Several cellular proteins have been identified as substrates of the ICE family of cysteine proteases (reviewed in [19]). These include poly(ADP-ribose) polymerase (PARP), an enzyme required for DNA repair and maintenance of genome integrity, which is cleaved primarily by CPP32 and Mch3 α [5,11,20], and nuclear lamins which are cleaved by Mch2 α [21,22].

Although there are exceptions [23,24], apoptosis induced by DNA-damaging agents such as etoposide and ionizing radiation, is primarily mediated by the nuclear phosphoprotein p53 [25,26]. Wild-type p53 is classified as a tumour suppressor gene because loss of this protein is a feature of more than half of all human tumours [27] and because mice that have no functional p53 gene rapidly develop multiple lesions [28]. DNA-damaging agents lead to the accumulation of wild-type p53 by post-translational stabilization [29] causing either cell cycle arrest at G₀/G₁, which facilitates DNA repair [30], or, in some systems, the induction of apoptosis [31].

One of the best models for studying mechanisms of p53 action

is the temperature-sensitive cell line, LTR6. LTR6 cells are a subclone of the murine myeloid leukaemic M1 cell line, stably transfected with a gene encoding a temperature-sensitive p53 protein. The transfected protein behaves as the mutant protein at 37.5 °C and as the wild-type protein at 32.5 °C. Cells shifted from 37.5 to 32.5 °C show no evidence of a cell cycle arrest [32] but instead rapidly lose viability and undergo apoptosis [31]. Although wild-type p53 leads to the induction of apoptosis in some systems, there has been no evidence to link wild-type p53 induction with the activation of ICE-like proteases. Using the LTR6 cell line we provide direct evidence for the activation of CPP32 and Mch3 α , and indirect evidence for the activation of Mch2 α . Our data support a mechanism of p53-induced apoptosis involving the sequential activation of members of the ICE family of cysteine proteases.

MATERIALS AND METHODS

Media and serum were purchased from Gibco (Paisley, Scotland, U.K.). Benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD.AFC) was from Enzyme Systems Inc. (Dublin, CA, U.S.A.). Acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD.AMC) and all other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Cell culture

The murine myeloid leukaemic cell lines M1 (parental; p53 null), LTRphe132 (non-temperature-sensitive mutant p53) and LTR6 (temperature-sensitive mutant p53) were generously given by Professor Moshe Oren (Weizmann Institute, Rehovot, Israel). All three cell lines were cultured in RPMI 1640 medium with L-glutamine, supplemented with 10% heat-inactivated foetal-calf serum in CO₂/air (1:19) and at 37.5 °C [31]. For the temperature shift, cells were transferred to a second incubator maintained at 32.5 °C.

Abbreviations used: Ac-YVAD.AMC, acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin; ICE, interleukin-1 β -converting enzyme; PARP, poly(ADP-ribose) polymerase; Z-DEVD.AFC, benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin.

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Flow cytometry and fluorescence microscopy

Flow-cytometric analysis was used to quantify apoptosis as described previously, with minor modifications [33]. The cells were stained at 37 °C for 10 min in the presence of 1.8 μ M Hoechst 33342. Propidium iodide (7.5 μ M) in PBS was added and the cells were analysed by flow cytometry (Becton Dickinson FACS Vantage), or examined by fluorescence microscopy.

Conventional agarose-gel electrophoresis

After the appropriate treatment, 0.75×10^6 cells/lane were applied on to a 1.8 % agarose gel and separated by electrophoresis. The gels were analysed for the presence of internucleosomal cleavage as described previously [33].

Western blot analysis

After the appropriate treatment, cells were subjected to SDS/PAGE and Western blotting as described previously [34]. The membranes were probed using a rabbit polyclonal antibody to the p17 subunit of CPP32 (kindly provided by Merck Frosst, Canada), a rabbit polyclonal antibody to PARP (318) (kindly provided by Dr. G. G. Poirier, Laval University, Quebec, Canada), a mouse monoclonal antibody to lamin B₁ (Serotec Ltd., Oxford, U.K.) and a rabbit polyclonal antibody raised to the p17 fragment of recombinant Mch3 α . Western-blot analysis verified that the antibody to Mch3 α recognized both intact Mch3 α (~36 kDa) and the p19 subunit, but did not recognize CPP32 (M. MacFarlane, K. Cain, X.-M. Sun, E. S. Alnemri and G. M. Cohen; unpublished work). Detection was achieved using the appropriate secondary antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham Life Sciences, Little Chalfont, Bucks., U.K.).

Lysate preparation and fluorometric measurement of proteolytic activity

Lysates from LTR6 cells were prepared as described previously for other cell lines [34]. The proteolytic activity of LTR6 cell lysates was measured using a continuous fluorimetric assay modified from the method of Thornberry [35]. Cleavage of Z-DEVD.AFC, which mimics the CPP32/Mch3 α cleavage site within PARP, and Ac-YVAD.AMC, which mimics the ICE cleavage site within proIL1 β , releases the fluorescent moieties 7-amino-4-trifluoromethylcoumarin (AFC) and 7-amino-4-methylcoumarin (AMC) respectively. This allows the quantitative analysis of any CPP32/Mch3 α - and ICE-like proteolytic activity. Liberation of AFC and AMC was measured using excitation wavelengths of 400 and 380, and emission wavelengths of 505 nm and 460 nm respectively. Lysates in 1.25 ml of 100 mM Hepes/10 % (w/v) sucrose/0.1 % (w/v) CHAPS/10 mM dithiothreitol, pH 7.5, were assayed at 37 °C in a modified cuvette holder fitted with a thermostat (Perkin-Elmer LS50B luminescence fluorimeter). Routinely, assays contained 15 μ g of lysate protein and 20 μ M substrate.

RESULTS

Wild-type p53 induces apoptosis in murine LTR6 cells

The murine LTR6 cell line contains a temperature-sensitive p53 protein that shows mutant characteristics at 37.5 °C and is indistinguishable from the wild-type protein at 32.5 °C [31,36]. Cells transferred to 32.5 °C for 22 h showed apoptotic characteristics, such as chromatin condensation and increased internucleosomal cleavage, which accorded with previous studies (Figures 1A and 1B; [31]). To obtain a quantitative rather than

qualitative assessment of p53-induced apoptosis in these cells, a modified flow-cytometric technique was used [33]. When temperature-shifted LTR6 cells were examined by flow cytometry, two distinct populations were observed: an apoptotic population of cells with high Hoechst 33342 fluorescence and a normal population with low Hoechst 33342 fluorescence. In contrast, no apoptotic population was detected in cells retained at 37.5 °C for 22 h (Figure 1C). To confirm that the apoptotic effects reported here were induced by the change in p53 status, two related cell lines, LTRphe132 and M1, were studied. LTRphe132 cells contain a non-temperature-sensitive mutant p53 and the M1 cells are the p53 null cell line from which both mutant lines are derived [31]. When LTRphe132 and M1 cells were transferred to 32.5 °C for 22 h, the cells retained normal morphology and displayed no chromatin condensation, internucleosomal cleavage or increase in Hoechst 33342 fluorescence (results not shown). Therefore the apoptotic changes observed in the LTR6 cells were a consequence of wild-type p53 induction.

Activation of CPP32 and Mch3 α in wild-type-p53-induced apoptosis

Cysteine proteases showing extensive structural and functional similarity to *Ced3* have been shown to play a pivotal role in mammalian apoptosis induced by a wide variety of stimuli. To determine whether members of this family of cysteine proteases play a role in p53-induced apoptosis, LTR6 cells were incubated at 32.5 °C or retained at 37.5 °C for up to 22 h. Cells transferred to 32.5 °C and assessed by flow cytometry showed a time-dependent increase in apoptosis, which increased significantly after 14–16 h at the permissive temperature (Figure 2). Cells retained at 37.5 °C for 22 h showed a basal level of apoptosis of approx. 9 % when assessed by this method (Figure 2). Using antibodies raised to the p17 subunits of CPP32 and Mch3 α , Western blots were used to look for evidence of processing of these two ICE homologues. After approx. 14 h at the permissive temperature, loss of the pro-forms of both CPP32 and Mch3 α was detected, concomitant with the appearance of the p17 and p19 cleavage fragments of CPP32 and Mch3 α respectively (Figure 2). Therefore the processing of both CPP32 and Mch3 α accompanied p53-induced apoptosis in LTR6 cells.

p53-induced apoptosis in LTR6 cells is accompanied by cleavage of identified substrates of ICE-like proteases

To assess further the involvement of ICE-like proteases in p53-induced apoptosis, cleavage of two cellular proteins, PARP and lamin B₁, known to be substrates for this family of cysteine proteases, was examined. The induction of wild-type p53 resulted in the cleavage of PARP into its signature apoptotic fragments after approx. 10–12 h, and provided further evidence for the presence of CPP32/Mch3 α -like proteolytic activity within these cells (Figure 3). Wild-type p53 induced the cleavage of lamin B₁ but this was not evident until after 16 h at the permissive temperature (Figure 3). This result is in agreement with previous observations that lamin cleavage is a relatively late event in the apoptotic pathway, occurring after the degradation of PARP [37,38]. The cleavage of lamin B₁ in LTR6 cells provided indirect evidence of Mch2 α activity induced by wild-type p53, as Mch2 α is the only ICE-like protease identified that is able to cleave lamins [21,22]. Thus p53-induced apoptosis in LTR6 cells is accompanied by the cleavage of cellular proteins known to be substrates of CPP32, Mch3 α and Mch2 α .

Figure 1 Wild-type-p53-induced apoptosis in LTR6 cells

LTR6 cells were transferred to 32.5 °C (lower panels) or retained at 37.5 °C (upper panels) for 22 h. (A) Cells were double-stained with Hoechst 33342 and propidium iodide and examined by fluorescence microscopy. (B) Cells were analysed for formation of internucleosomal DNA fragments by conventional agarose-gel electrophoresis. (C) Cells were double-stained as in (A) and analysed by flow cytometry. The numbers in each region of the cytogram represent the percentage of cells exhibiting basal and increased Hoechst 33342 fluorescence. The results shown are from a typical experiment and demonstrate that cells transferred to 32.5 °C for 22 h exhibit apoptotic morphology, increased internucleosomal cleavage of DNA and increased Hoechst 33342 fluorescence.

Figure 2 Wild-type-p53-induced processing of CPP32 and Mch3 α

LTR6 cells were incubated for up to 22 h at 32.5 °C (lanes 2–9) or retained at 37.5 °C (lane 1; control). Lanes are numbered from the left. The percentage of apoptotic cells at each time point (determined by flow cytometry) is indicated above the appropriate lane. Samples were taken from the same experiment to determine the extent of processing of both CPP32 and Mch3 α . Cellular proteins were separated by SDS/13% (w/v) PAGE, and Western blotting was carried out using antibodies to CPP32 (upper panel) and Mch3 α (lower panel). The proform (upper arrowheads) and major subunit (lower arrowheads) of each protease are indicated. Molecular-mass markers are indicated in kDa on the left.

Figure 3 Wild-type p53 induces PARP and lamin B₁ cleavage

LTR6 cells were incubated for up to 22 h at 32.5 °C (lanes 2–9) or retained at 37.5 °C (lane 1; control). Lanes are numbered from the left. Cellular proteins were separated by SDS/7% (w/v) PAGE (PARP) or SDS/10% (w/v) PAGE (lamin B₁) and Western blotting was carried out to assess the incidence of PARP cleavage (upper panel) and lamin B₁ cleavage (lower panel). The intact substrates (upper arrowheads) and their cleavage fragments (lower arrowheads) are indicated. Molecular-mass markers are indicated in kDa on the left.

Table 1 Increased Z-DEVD.AFC cleavage activity in temperature-shifted LTR6 cell lysates

LTR6 and LTRphe132 cells were transferred to 32.5 °C or retained at 37.5 °C for 22 h. Lysates prepared from these cells were then assayed for Z-DEVD.AFC cleavage activity as described in the Materials and methods section. The specific activities of control lysates (37.5 °C) and lysates prepared from temperature-shifted cells (32.5 °C) are shown. The values represent the means $\pm$ S.E.M. for three experiments.

Temperature °C	Specific activity (pmol/min per mg of protein)	
	LTR6	LTRphe132
37.5	130 $\pm$ 60	30 $\pm$ 15
32.5	3580 $\pm$ 1220	90 $\pm$ 10

Lysates prepared from apoptotic LTR6 cells exhibit Z-DEVD.AFC cleavage activity

To characterize the proteolytic activity in lysates prepared from temperature-shifted LTR6 cells, Ac-YVAD.AMC, a model substrate for ICE activity was used to determine whether any activation of ICE had occurred in temperature-shifted cells. No hydrolysis of Ac-YVAD.AMC was detected in lysates obtained from either control cells or cells shifted to the permissive temperature for 22 h (results not shown), suggesting that ICE may not be activated by wild-type p53. Others have reported a peak of ICE-like proteolytic activity preceding a CPP32/Mch3 α -like activity [39]; therefore, at this stage, we cannot exclude the possibility that there is a small but transient increase in ICE activity which may occur before 22 h. In marked contrast, using Z-DEVD.AFC, which mimics the CPP32/Mch3 α cleavage site within PARP, lysates from temperature-shifted LTR6 cells showed at least a 28-fold increase in Z-DEVD.AFC cleavage activity above that seen in lysates prepared from control LTR6 cells (Table 1). Processing of the pro-forms of both CPP32 and Mch3 α was detected in lysates prepared from temperature-shifted LTR6 cells, but not in lysates prepared from control LTR6 cells, confirming that the cleavage of CPP32 and Mch3 α resulted in a proteolytic cleavage activity characteristic of both homologues (results not shown). Lysates prepared from both control and temperature-shifted LTRphe132 cells (which contain a non-temperature-sensitive mutant p53) displayed levels of Z-DEVD.AFC cleavage activity similar to that seen in control LTR6 cell lysates (Table 1). In agreement with this, no cleavage of CPP32, Mch3 α , PARP or lamin B₁ was detected in LTRphe132 cells shifted to the permissive temperature for 22 h (results not shown). This confirmed that wild-type p53, and not the temperature shift *per se*, had induced CPP32/Mch3 α -like proteolytic activity in temperature-shifted LTR6 cell lysates.

DISCUSSION

DNA-damaging agents are well documented as inducers of apoptosis (reviewed in [40]). A number of these agents including etoposide, UV light and ionizing radiation induce apoptosis primarily via a p53-dependent mechanism [29]. DNA damage induced by these agents results in the accumulation of wild-type p53 by post-translational stabilization causing either cell-cycle arrest or apoptosis. Apoptosis was induced in LTR6 cells containing a temperature-sensitive mutant p53 transgene, by incubation at the permissive temperature for up to 22 h (Figure 1; [31]). Although the precise biochemical effects mediated by p53 in apoptosis have yet to be determined, wild-type p53

directly *trans*-activates and *trans*-represses transcription from the *bax* and *bcl-2* promoters respectively [41], resulting in a high Bax:Bcl-2 ratio that favours apoptosis [42]. However, whether this is a crucial event required for apoptosis in LTR6 cells is not clear.

To date there have been no reports demonstrating a direct role for ICE-like proteases in p53-induced apoptosis. In this report we show that apoptosis induced by wild-type p53 in LTR6 cells is accompanied by the processing and activation of CPP32 and Mch3 α , together with the proteolysis of their common substrate PARP (Figures 2 and 3). Our results suggest that the cleavage of PARP is a relatively early event, which precedes the onset of apoptosis as assessed by flow cytometry, and occurs before the processing of both CPP32 and Mch3 α is detectable. Although we cannot rule out a difference in sensitivity between the PARP antibody and the antibodies to CPP32 and Mch3 α , our results are consistent with those of others who report the activation of a PARP-cleaving homologue before the activation of CPP32 [43].

Lysates prepared from temperature-shifted LTR6 cells showed a 28-fold increase in Z-DEVD.AFC cleavage activity compared with lysates prepared from control cells. Therefore p53-induced apoptosis in LTR6 cells is associated with the generation of a Z-DEVD.AFC cleavage activity (Table 1) which is attributable to members of the CPP32 subfamily of ICE-like proteases, namely CPP32, Mch3 α and Mch2 α . Consistent with this are our observations that CPP32 and Mch3 α are processed in lysates prepared from temperature-shifted, but not control, LTR6 cells (results not shown). However, in addition to CPP32, Mch3 α and Mch2 α , we cannot exclude the possibility that the increase in Z-DEVD.AFC cleavage activity observed in temperature-shifted LTR6 cells may be due, in part, to another as yet unidentified CPP32 subfamily member(s) that also cleaves PARP.

Besides the activation of CPP32 and Mch3 α during wild-type p53-induced apoptosis, we also report the p53-induced cleavage of lamin B₁. Lamin cleavage occurs after the occurrence of PARP cleavage (Figure 3, bottom panel), in agreement with previous studies [37,38]. To date, Mch2 α is the only ICE homologue reported to be capable of cleaving nuclear lamins [21,22]. The cleavage of lamin B₁ in our system provides indirect evidence for the involvement of a third ICE homologue, Mch2 α , in p53-induced apoptosis. However, we cannot exclude the possibility that the proteolytic cleavage of lamin B₁ in LTR6 cells may also be mediated by a Ca²⁺-regulated serine protease associated with the nuclear scaffold [44].

ICE-like proteases have been implicated in the execution phase of apoptosis induced by a wide variety of stimuli (reviewed in [19]), including Fas-induced apoptosis in Jurkat T-cells [34,45]. Studies of Fas receptor-mediated apoptosis have recently provided the first direct link between a receptor-linked signalling pathway and the effector mechanisms of apoptosis. Ligation of the Fas receptor activates MACH/FLICE/Mch5, a recently identified member of the ICE family of proteases [15–17]. The emerging tenet from all of these studies is that widely diverse stimuli initiate apoptosis through specific mechanisms, but that the execution phase involves the sequential activation of an amplifying cascade of ICE-like proteases [13,17,38,39]. In agreement with these observations, the p53-induced apoptosis described here provides a further example of an apoptotic stimulus that feeds into the common apoptotic execution pathway involving the sequential activation of ICE-like proteases.

The results of the present study provide a direct link between p53-induced apoptosis and the activation of members of the ICE-family of proteases. We have shown that p53-induced apoptosis is accompanied by the processing and activation of

CPP32, Mch3 α and a lamin protease, together with the cleavage of their substrates, PARP and lamin B₁. In summary, our data support a functional role for the sequential activation of ICE-like proteases in the execution phase of p53-induced apoptosis.

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