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Transcriptional repression by p53 promotes a Bcl-2-insensitive and mitochondria-independent pathway of apoptosis

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ABSTRACT

p53 can induce apoptosis in various ways including transactivation, transrepression and transcription-independent mechanisms. What determines the choice between them is poorly understood. In a rat embryo fibroblast model, caspase inhibition changed the outcome of p53 activation from standard Bcl-2-regulated apoptosis to caspase-independent and Bcl-2-insensitive cell death, a phenomenon not described previously. Here, we show that caspase inhibition affects cell death commitment decisions by modulating the apoptotic functions of p53. Indeed, in the Bcl-2-sensitive pathway, transactivation-dependent signalling is activated leading to a rapid MDM2-mediated degradation of p53. In contrast, in the Bcl-2-insensitive pathway, p53 is stable and this is associated with transrepression-dependent signalling. A study with microarrays identified these genes regulated by p53 in the absence of active caspases.

INTRODUCTION

The balance between cell proliferation and apoptosis is crucial for the normal development and tissue-size homeostasis in adult mammals. One of the most important links between the proliferation and cell death machineries is the tumour suppressor protein p53. This protein promotes cell-cycle arrest or apoptosis in response to DNA damage or strong oncogenic stimuli for proliferation (1). p53 is a phosphoprotein that is pivotal in suppressing cellular transformation and tumorigenesis. In about half of the human cancers, p53 is inactivated directly as a result of mutations in the *p53* gene. In many others, it is inactivated indirectly through binding to viral proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53.

The amount of p53 in cells is principally determined by the rate at which it is degraded. The sequence-specific transcriptional induction of the *mdm2* gene results in a product, MDM2,

which binds to p53 and stimulates the addition of ubiquitin groups to the C-terminus of p53. The ubiquitinated p53 is detected and degraded by the proteasome (2). In normal cells, p53 gene expression is low. In stress conditions or following DNA damage, post-translational changes in p53 or MDM2 can disrupt the balance and allow p53 activation. p53 can be modified by phosphorylation, acetylation, glycosylation or addition of ribose, and these events can regulate p53 function (1).

p53 action is a transcriptional activator role, forming tetramers that bind DNA in a sequence-specific manner by using a highly conserved DNA-binding domain. The transcriptional targets of p53 include genes implicated in cell cycle regulation, including *p21*, *gadd45* and *14-3-3σ* (3,4). p53 also induces the transcription of proapoptotic genes, such as *fas*, *nox*, *killer/dr5* and *bax*, which leads to caspase activation with the help of death-inducing signalling complex (at the cell membrane level) or the apoptosome (at the mitochondrial level) (5–7).

In many models, the transactivation function is not essential for p53-dependent apoptosis (8–10) but the transrepression function may be important (11,12). Indeed, p53 represses the transcription of a number of genes, including those involved in regulatory cascades mediating cell proliferation (*cyclin B* and *RNA polymerase I*) and apoptosis (*bcl-2*). Furthermore, p53-mediated repression of genes involved in cytoskeleton organization (*stathmin* and *map4*), leading to a decrease in microtubule polymerization, participates in both cell cycle arrest and apoptosis (13). In contrast to the information available concerning p53 as an activator, the mechanism by which repression occurs is less well documented (14,15). This is due in a large part to there being no identified consensus p53-binding site within repressed promoters (16,17). The genes up- and down-regulated by p53 are not the same; induced genes being principally pro-apoptotic and repressed genes being anti-apoptotic or important for cell survival. It has been shown that p53 can lead to transcription-independent caspase activation (18), probably through a direct effect on the release of apoptogenic molecules from mitochondria (19). However, the physiological relevance of this apparently

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redundant pro-apoptotic property and the determinism of choice between them are unknown.

We have previously shown that large tumour (LT) inactivation leads to p53-mediated apoptosis in rat embryo fibroblasts (e.g. the REtsAF cell line) expressing a temperature-sensitive mutant (tsA58) of the Simian Virus 40 (SV40) LT antigen (20,21). Moreover, whereas *bcl-2* overexpression inhibits apoptosis, caspase inhibition surprisingly both accelerates apoptosis and abolishes the protective effect of Bcl-2 (22). Z-Val-Ala-DL-Asp-Fluoromethylketone (ZVAD)-mediated caspase inhibition changed the outcome of p53 activation from Bcl-2-regulated apoptosis to mitochondria- and caspase-independent cell death, a phenomenon that had not been described previously (23).

Indeed, even though it is clear today that physiological cell death can occur in the complete absence of caspases, only a few cases of apoptotic death without caspase activation have been reported, most often caspase-independent cell death is related to paraptosis, autophagy or non-lysosomal cell death (24,25). Moreover, mitochondrial outer membrane permeabilization (MOMP) controlled by Bcl-2 family proteins resides at the heart of several alternative death pathways whatever be their apoptotic or necrotic feature. Therefore, the p53-induced cell death program in the presence of ZVAD appears to differ from most caspase-independent alternative pathways: on the one hand by its apoptosis-like nature and on the other hand by being MOMP-independent and insensitive to Bcl-2 protection.

Here, we show that ZVAD treatment affects the cell death commitment decision by modulating the apoptotic functions of p53. Indeed, in the absence of a caspase inhibitor, p53 activation promotes Bcl-2-sensitive apoptosis through transactivation-dependent signalling that is associated with a rapid MDM2-mediated degradation of p53. In contrast, caspase inhibition triggers a Bcl-2-insensitive pathway involving the stabilization of p53 and transrepression-dependent signalling. A global study of the transcriptome led to the identification of genes differentially regulated by p53 according to these two signalling pathways.

MATERIALS AND METHODS

Cell lines, cell culture and drugs

A rat embryo (RE cells) fibroblast culture was infected with a variant of the SV40, which expresses a temperature-sensitive mutant of the LT antigen, tsA58 (26), and the temperature-sensitive REtsAF cell line was selected and isolated at 33°C. The wild-type LT immortalizes these cells via the inhibitory binding of the p53 tumour suppressor. However, the tsA58 mutant of LT cannot interact with p53 at restrictive temperature. Thus, the cells are immortalized at permissive temperature (33°C), whereas at restrictive temperature (39.5°C) heat-inactivation of LT leads to the release of p53, promoting the commitment to apoptosis (20,26).

The REtsAF-Bcl-2 cell line has been described previously (22,27). In this cell line, *bcl-2* overexpression is under the control of tetracycline (Tet-off system). These cells were propagated at 33°C in DMEM (Invitrogen) supplemented with 100 µg/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), 1% Glutamax (Invitrogen) and 10% foetal calf serum (Invitrogen), under 5% CO₂/95% air. REtsAF-Bcl-2

cells were maintained in the absence of tetracycline for one week before the experiments described below to allow the expression and the accumulation of exogenous *bcl-2*. For inhibitor treatments, ZVAD (Bachem), a broad-spectrum caspase inhibitor was used at a concentration of 100 µM.

Western-blot analysis

The cells were seeded in 100 mm dishes and incubated at 33°C until they reached 70% confluence. The dishes were then incubated at 39.5°C for various time periods in the presence or absence of 2 µg/ml tetracycline and/or 100 µM ZVAD. The cells were rinsed in cold phosphate-buffered saline, collected with a scraper and frozen at -20°C. Proteins (80 µg) were separated by SDS-PAGE (in 15% acrylamide/0.2% bisacrylamide to resolve Bax and p21 and in 7.5% acrylamide/0.1% bisacrylamide for p53 and MDM2) and transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Boehringer Mannheim) (28). Blots were exposed to the first antibody diluted in Tris-buffered saline (TBS)/5% milk overnight at 4°C, rinsed in TBS/0.5% Tween-20 and exposed for 1 h, at room temperature, to horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat immunoglobulin serum (Biosystem) as appropriate for the first antibody used. Blots were washed in TBS, and the immunoreactive bands were revealed using the Amersham ECL[®] kit. The antibodies used were rabbit polyclonal anti-Bax (N-20; Santa Cruz), goat polyclonal anti-p21 (C-19; Santa Cruz), mouse monoclonal anti-p53 (Pab 122; gift from Dr E. May, IRSC, Villejuif, France), mouse monoclonal anti-LT (Pab 416; gift from Dr E. May, IRSC, Villejuif, France) and mouse monoclonal anti-MDM2 (SMP40; Santa Cruz Biotechnology). All blots were normalized by reference to rat monoclonal anti-tubulin (MAS078; Sera-Lab) binding.

mRNA detection

At 70% confluence, REtsAF-Bcl-2 cells, overexpressing or not overexpressing *bcl-2*, were incubated in the presence or absence of 100 µM ZVAD for various time periods at restrictive temperature (39.5°C). RNA was isolated by the guanidium isothiocyanate method (29). mRNA was assayed either by northern-blot analysis or by RT-PCR.

Northern-blot analysis. An aliquot of 20 µg of total RNA was separated by electrophoresis on a 1.25% agarose gel containing 0.66 M formaldehyde and was transferred onto a Hybond-N⁺ membrane (Amersham). mRNA was detected by hybridization with specific probes (stathmin, map4 and 18S RNA) produced by PCR amplification and ³²P-labelled by the megaprime DNA-labelling system RPN 1606 (Amersham).

RT-PCR assay. To determine the levels of bax, p21 and mdm2 mRNAs, RT-PCR was used as described previously (30) with the specific primers listed in Table 1. A total of 20–30 PCR cycles were performed, according to the amount of mRNA. In all cases, synthetic tobacco leaf nitrate reductase (NR) transcripts were co-reverse-transcribed and co-amplified with the samples as an efficiency control (30). Amplified products were separated on a 10% acrylamide gel, stained with ethidium bromide, photographed with a SynGene GeneStore system and bands quantified with ImageQuant software.

Table 1. Sequences of primers used in RT-PCR experiments

Specificity	Orientation	Sequence (5'→3')	PCR fragment size (bp)
Bax	Antisense	TTCTTGGTGGATGCATCCT	158
	Sense	GGAGCAGCTCGGAGGCG	
p21	Antisense	TGAATGAAGGCTAAGGCAGAAGA	146
	Sense	AGGCAGACCAGCCTAACAGATT	
Mdm2	Antisense	ATTCCACACTCTCGTCTTTGTC	75
	Sense	AGCATTGTTTATAGCAGCCAAGAA	
NR	Antisense	GCTGGATCCATTGCAAATTCC	77
	Sense	AGGAGCTGATGTGTTGCCCGG	

DNA chip analysis

Microarrays. Chips were designed and used at the microarray platform of the 'service de génomique fonctionnelle' in CEA of Evry (France). The chips were a development of chips described previously by Preisser *et al.* (31) and Delmar *et al.* (32), and contain 7330 probes. Each probe corresponds to a particular gene and some genes are spotted two to five times, such that there is redundancy which is useful to evaluate the relevance of results. These probes contain 2014 PCR products amplified from a cDNA matrix using primers specific for genes involved in important biological processes, including apoptosis, cell cycle and the stress response. Chips were enriched with 1684 mouse cDNA clones from the IMAGE consortium (Research Genetics collection), 1500 mouse cDNA (named SDD and SDM) clones of a subtractive bank (myodistrophic versus normal muscle) and 1800 clones of rat cDNA (named RDD and RDM) from another subtractive bank (atrophied versus normal muscle). Each insert was amplified by PCR with specific primers. PCR products were 400–2000 bp long. All PCR products were prepared in 96-well plates, purified by ethanol precipitation, washed in 70% ethanol, dried, dissolved in TE/DMSO (50/50) and stored at -20°C . Quality, size and concentration of these PCR products were determined by electrophoresis. Typically, the DNA concentration was between 50 and 300 ng/ μl . Gels were analysed using the Genetools software (Syngene, Merck Eurolab, Fontenay-sous-Bois, France) and size, concentration and quality were automatically annotated in the file, which was included in the final differential expression data file. The PCR products were arrayed on CMT-CAPSTM slides (Corning) using a Microgrid-II (Biorobotics). Arrayed slides were stored in a dark dry place at room temperature until use. Just before hybridization, spotted DNA was cross-linked to the support by UV irradiation with a total energy of 260 mJ (254 nm).

Total RNA extraction and cDNA labelling. At 70% confluence, REtsAF-Bcl-2 cells were incubated at 39.5°C in the presence or absence of 100 μM of ZVAD for 16 h. RNA was isolated by the guanidium isothiocyanate method (29). RNA concentrations were assessed by optical density measurements, and the quality was evaluated by electrophoresis and with a Bio-analyser RNA nano assay (Agilent). Modified cDNA was prepared with the reverse transcriptase superscript 2 (Gibco-BRL Life Technologies) according to the manufacturer's protocol, in the presence of 20 μg of total RNA, 1 ng of luciferase mRNA (Promega) as a control, 4 μg of oligo(dT) (Amersham Pharmacia Biotech), dUTP aminoallyl (Sigma), dNTP mix

(Roche) (2 mM each except 1.6 mM aminoallyl dUTP and 0.4 mM dTTP) to obtain a aminoallyl-dUTP/dTTP ratio of 4:1). cDNA was indirectly labelled by incubating modified cDNA with NH_2 -cyanine 5 or NH_2 -cyanine 3 in 0.1 M sodium bicarbonate buffer for 30 min at room temperature. Then, to suppress cross-hybridization to repetitive DNA, 10 μg of Cot-1 cDNA (Gibco-BRL Life Technologies), 10 μg of poly(A) (Amersham Pharmacia Biotech) and 10 μg of yeast tRNA (Gibco-BRL Life Technologies) were added to the labelled cDNA. Samples were centrifuged for 1 h at 4°C at 13 000 r.p.m. and the resulting pellet was dissolved in 60 μl of hybridization buffer [50% formamide (Sigma-Aldrich), 2.5 \times Denhart (Sigma-Aldrich), 0.5% SDS (Gibco-BRL Life Technologies) and 6 \times SSPE (Gibco-BRL Life Technologies)].

Hybridization. RNA obtained from cells cultured in the absence of active p53 (33°C) was used as a control throughout the experiments to allow comparisons between samples. Each experiment was repeated four times, for optimal reproducibility, and the dye swap procedure was used (i.e. once with the test sample labelled with Cy3 and the control labelled with Cy5, and once with control stained with Cy3 and the test sample labelled with Cy5). Samples were denatured for 2 min at 100°C and hybridizations were performed in specific chambers (Corning) overnight at 42°C . Coverslips (Sigma-Aldrich) were then removed by immersing the slide in 2 \times SSC, 0.1% SDS buffer for 15 min and twice in 0.1 \times SSC for 15 min at room temperature on an orbital shaker. Slides were dried by centrifugation for 5 min at 700 r.p.m.

Image acquisition and microarray analysis. Slides were scanned on a Packard Scan Array Express with 10 μm resolution and the TIFF images generated were imported into GENEPiX pro 4 software (Axon). This software was used for the alignment of the image with the grid, spot detection and extraction of Cy5 and Cy3 intensities for all spots. Analysis files were then imported into the GENESPRING[®] 6.1 software (Silicon Genetics). The first stage of the analysis was to normalize data from each slide by the 'lowess' method (with a window of 40%) (33,34). Then, the minimal intensity value needed to obtain relevant data was evaluated by GENESPRING[®] software: a value of 477.4 U was obtained. This step is important because lower intensity values are not reproducible and have high standard deviations (G. Gruel, C. Lucchesi, A. Pawlik, O. Alibert, V. Frouin, T. Kortulewski, X. Gidrol and D. Tronik-Le Roux, manuscript in preparation). Only spots having an intensity value above this threshold in all the three conditions (33°C , 39.5°C and 39.5°C + ZVAD) and in all four experiments were considered. As each experiment was repeated four times, we did not use the global error model for the GENESPRING[®] analysis. Finally, 3513 probes giving statistically reproducible results were included in the study.

To adjust for any bias arising from variations in the microarray technology, a self-self experiment was performed in which two identical mRNA samples were labelled with different dyes and hybridized to the same slide. The experiment was repeated four times, and two independently prepared samples from the 33°C condition were used. Only 0.85% of genes (30/3513 probes) were found to vary under this condition ($P > 0.05$; $n = 4$) confirming both the reproducibility and the robustness of our method. Expression ratios of these

modulated genes do not exceed 0.73 for repressed genes and 1.39 for activated genes.

To identify modulated genes, we applied the limits determined in the self-self experiment (see previous paragraph) rather than arbitrary limits with no biological relevance. These limits were selected because no gene's expression varies beyond them in a random manner. Ratios between 0.73 and 1.39 are then not considered in this work. The *t*-test ($P < 0.05$, $n = 4$) was also used to ensure that all presented ratios are significantly different from 1.

RESULTS

p53 protein is stabilized in the presence of ZVAD

We investigated whether ZVAD-dependent regulation of apoptosis is associated with changes in p53 status. We first assayed p53 by western blotting at permissive temperature (33°C) and at various time periods after a temperature shift to 39.5°C (Figure 1). The p53 detected at permissive temperature was in the inactive state, bound to SV40 LT antigen. After the temperature shift in the absence of ZVAD, there was a rapid, time-dependent decrease in p53 levels. This is consistent with reports that p53 induces expression of the *mdm2* gene, the product of which induces degradation of p53 by the proteasome. Conversely, no decrease in p53 abundance was observed in the presence of ZVAD. RT-PCR analysis showed that ZVAD treatment did not affect the amount of p53 transcript (data not shown), so the regulation of the p53 abundance by ZVAD seems to result from a post-transcriptional mechanism. We then examined the effect of ZVAD on the LT antigen (Figure 1): LT gave several bands in western blots due to the diverse modifications of the LT gene following its introduction into mouse or rat cell lines (35). We found that ZVAD did not significantly influence the amount of LT (Figure 1), indicating that p53 stabilization was not due to changes in LT levels.

These results suggest that ZVAD inhibits the degradation of p53 by the proteasome, resulting in a higher concentration of p53 as early as after 8 h at restrictive temperature. This coincides with the increased rate of apoptosis reported previously (22).

p53 can induce transcriptional activation-dependent or -independent apoptotic processes

The stabilization of p53 by ZVAD may lead to increased p53 transactivation activity of p53 and thereby explain the accelerated death process observed in this condition. To test

this hypothesis, mRNAs of three p53 target genes (*p21*, *bax* and *mdm2*) were assayed by RT-PCR. In the absence of ZVAD, p53 activation at 39.5°C increased the transcription of the three genes. Surprisingly, in the presence of ZVAD and thus more p53, there was less accumulation of these messengers (Figure 2A). To confirm that this effect was not a result of ZVAD directly, and independent of p53, we compared mRNA levels at permissive temperature in the presence and absence of ZVAD 24 h after addition (Figure 2A, in frame). The intensity of the *p21*, *bax* and *mdm2* mRNAs was very weak

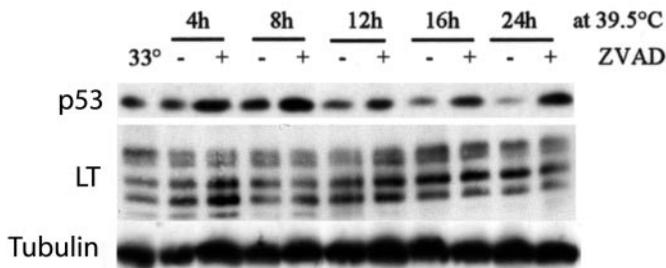


Figure 1. Effect of ZVAD treatment on p53 status. Western-blot analysis of p53 and AgT in REtsAF cells in the presence and absence of ZVAD. Time following the temperature shift to 39.5°C is indicated above the lanes. Tubulin was used as a loading control.

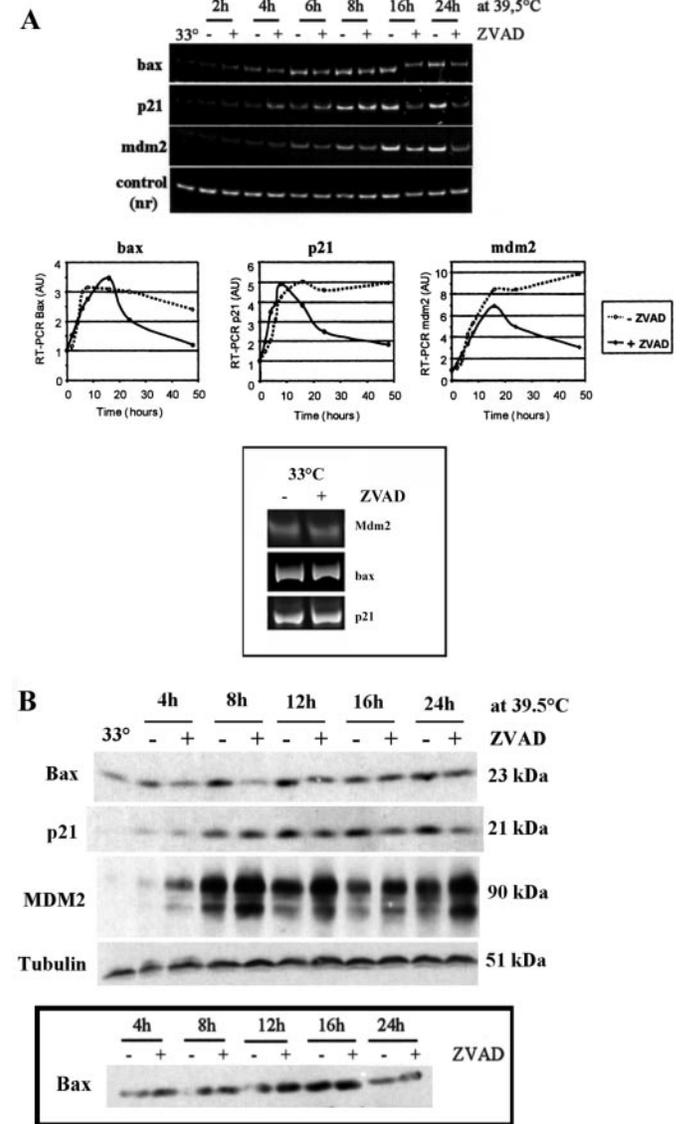


Figure 2. Effect of ZVAD treatment on the transcriptional activation function of p53. (A) Study of the transcriptional activation of p53 targets. *p21*, *bax* and *mdm2* mRNA were amplified by RT-PCR (upper panel). Time following the temperature shift to 39.5°C is indicated above the lanes. Amplification products were quantified using the ImageQuant software (lower panel). Units are arbitrary and represent the expression level of each gene as compared to that at permissive temperature. RT-PCR of synthetic transcripts of tobacco NR was used as control. Effect of ZVAD on the three targets at permissive temperature is shown in frame (B), western blot of *p21*, *Bax* and *MDM2* in the same conditions as in (A). *MDM2* and *p21* proteins are too weak in the absence of active p53 (33°C) to be detectable. Tubulin was used as a loading control. Effect of ZUAD on *Bax* protein at permissive temperature is shown in frame.

consistent with these genes being poorly expressed in the absence of active p53. Furthermore, there was no difference according to the presence or absence of ZVAD. ZVAD thus has no effect when p53 is inactive.

We tested whether the amount of protein corresponds to the amount of messenger by western blotting (Figure 2B). p21 and Bax proteins followed the abundance of their messengers: the proteins accumulated at restrictive temperature, both in the absence and presence of ZVAD, even though the increases observed in the presence of ZVAD were lower than those detected in the absence of ZVAD. Conversely, after an initial increase in both protein and messenger levels (until 12 h at 39.5°C), changes in the MDM2 protein level differed from those in the amount of messenger: in the absence of ZVAD, the amount of MDM2 gradually declined whereas messenger levels were maintained; in the presence of ZVAD, MDM2 levels were stable whereas the messenger decreased (cf. Figure 2A and B). This discrepancy between protein and messenger changes suggests post-transcriptional regulation of MDM2. We confirmed that, like for mRNA levels, ZVAD had no effect on Bax proteins in the absence of active p53 (Figure 2B, in frame). In this condition MDM2 and p21 proteins are too weak to be detectable.

Thus p53 activation at restrictive temperature induces the transactivation of target genes. This transactivation is attenuated in the presence of ZVAD despite a greater abundance of p53. MDM2 and p53 proteins present similar fates: the lower protein concentrations in the absence of ZVAD may be due to the ubiquitin ligase function of MDM2 for itself and p53 promoting proteasomal degradation; the stabilization of both p53 and MDM2, in the presence of ZVAD, might be a consequence of less efficient entry into the proteasome when caspases are inhibited.

p53-mediated transcriptional repression is involved in the caspase-independent apoptotic pathway

Surprisingly, p53 accumulation in the presence of ZVAD is associated with a reduced transactivation of its target genes. Therefore, we investigated whether the accelerated apoptosis induced by ZVAD involves the transcriptional repression activity of p53. We tested the transcription of the *map4* and *stathmin* genes in REtsAF-Bcl-2 cells by northern blotting. The products of these genes regulate microtubule polymerization, and their transcription is negatively regulated by p53 (13,36). The *map4* mRNA is subject to alternative splicing but the messenger studied was the full-length form (6.7 kb). We found that p53 activation at 39.5°C correlated with a transient decrease in both *map4* and *stathmin* mRNA levels and that the decrease was greater in the presence of ZVAD (Figure 3). This suggests that ZVAD-induced apoptosis results from an increased transrepression activity of p53. Also, regulation of the amount of p53, involving one or more caspase(s), may affect which p53 functions are involved: increased amounts of p53 are associated with greater transrepression versus transactivation activity.

Caspase inhibition promotes a large-scale decrease in p53-dependent transcriptional activity

Unlike genes whose activation by p53 induces apoptosis, little is known about genes whose repression by p53 contributes to

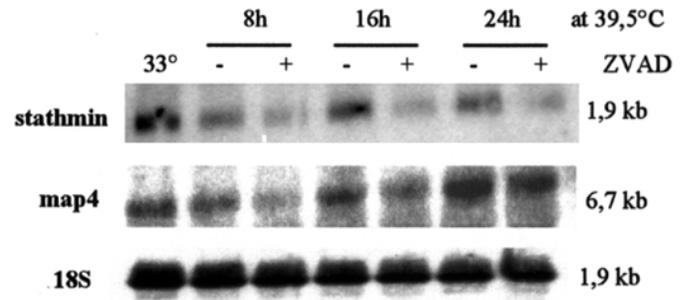


Figure 3. Effect of ZVAD treatment on the transcriptional repression function of p53. Northern-blot analysis of *stathmin* and *map4* mRNAs in REtsAF cells in the presence or in the absence of ZVAD. Time after temperature shift to 39.5°C is indicated above the lanes. 18S mRNA was used as a loading control.

apoptosis. We used DNA microarrays to identify such genes and to elucidate the signalling pathway activated by p53 in the presence of ZVAD.

Transcriptional regulation following p53 activation was assessed in the presence and in the absence of ZVAD. RNA was extracted from cells 16 h after the temperature shift to 39.5°C. At this stage, transcriptional modifications mostly reflect the onset of apoptosis rather than the cellular destruction process. RNA corresponding to both test conditions (active p53: 39.5°C, in the presence or in the absence of ZVAD) was hybridized against control RNA (inactive p53: 33°C). Each hybridization was performed four times with an inversion of fluorochromes to optimize reliability and to minimize staining artefacts, and the data were averaged.

Hybridization of RNA from cells cultured at 39.5°C with that from cells at 33°C identified 179 genes that were significantly activated (from 1.4- to 26-fold) and 214 are significantly repressed (from 1.4- to 10-fold) at restrictive temperature. They included many known p53-responsive genes (*p21*, *GADD45* and *IGFBP*). Other p53-responsive genes were absent either because they did not pass the filters (e.g. *bax*) or simply because they were not represented on the chips. However, similar microarray studies have shown previously that the pattern of expression of p53-target genes is dependent on p53 activation status; not all known p53 targets are simultaneously activated in any given condition (37–39). Many genes not previously identified as p53-direct-targets were also regulated by p53 induction. Some of them have been described in other similar studies, as *ATPase*, *H + transporting* and *hsp70* genes were repressed, and *amyloid protein precursor* and *annexin* genes were activated (37–39). Our data did not indicate whether these genes are primary or secondary transcriptional targets of p53.

In the presence of ZVAD (hybridization 39.5°C + ZVAD/33°C), 220 genes were activated and 385 were repressed. Thus, the numbers of activated genes are comparable in the presence and absence of ZVAD, but about twice as many genes are repressed in the presence of ZVAD than in its absence. Interestingly, 75% of the genes repressed in the absence of ZVAD were also repressed in the presence of the drug, although 215 more genes were repressed in the presence of the drug (see Venn diagram in Figure 4).

To identify genes that are specifically modulated by ZVAD, we used the ANOVA parametric test (on conditions 39.5°C/

39.5°C + ZVAD) with a $P < 0.05$. Moreover, among these genes, only those with ratios of the expression level at 39.5°C in the presence of ZVAD to that in the absence of ZVAD below 0.73 or above 1.39 were considered relevant.

Ninety-four genes were thereby identified as being significantly and specifically modulated (81 repressed and 13

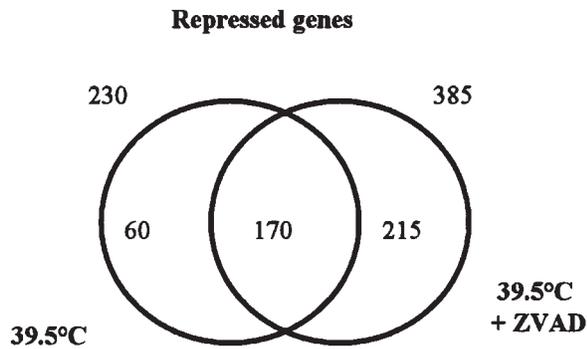


Figure 4. Venn diagram representation of repressed genes. The number of repressed genes in the absence of ZVAD is represented in the left circle. The number of repressed genes in the presence of ZVAD is represented in the right circle. Number of genes repressed in both the absence and presence of ZVAD is at the intersection of the two circles.

induced) when p53 is active in the presence of ZVAD. Some of these genes have not been identified because not all the probes have been sequenced. Interestingly, the few genes which were represented by more than one spot on the chip gave reproducible results. For example, the *procollagen type III alpha 1* gene appeared five times with the same negative regulation. These various *procollagen* probes allowed to estimate the experimental variation: it was 0.007 in the absence of ZVAD and 0.004 in the presence of ZVAD. This indicates the reproducibility of the results.

We classified the genes into four clusters according to their specific modulation after ZVAD addition (Figure 5 and Table 2): those which were less activated (cluster 1: 11 genes), more repressed (cluster 2: 68 genes), more activated (clusters 3: 10 genes) and less repressed (cluster 4: 3 genes). Most (81/94, 86%) were less expressed in the presence of ZVAD.

Note that genes sensitive to the temperature shift and not to p53 activation were excluded as their temperature-dependent modulations are the same in the presence or absence of ZVAD.

In conclusion, transcriptosome analysis indicates that the ZVAD-mediated commitment to an alternative Bcl-2-insensitive programme was associated with a shift from a transactivation function towards a transrepression activity of p53. This is consistent with the RT-PCR and northern-blot findings.

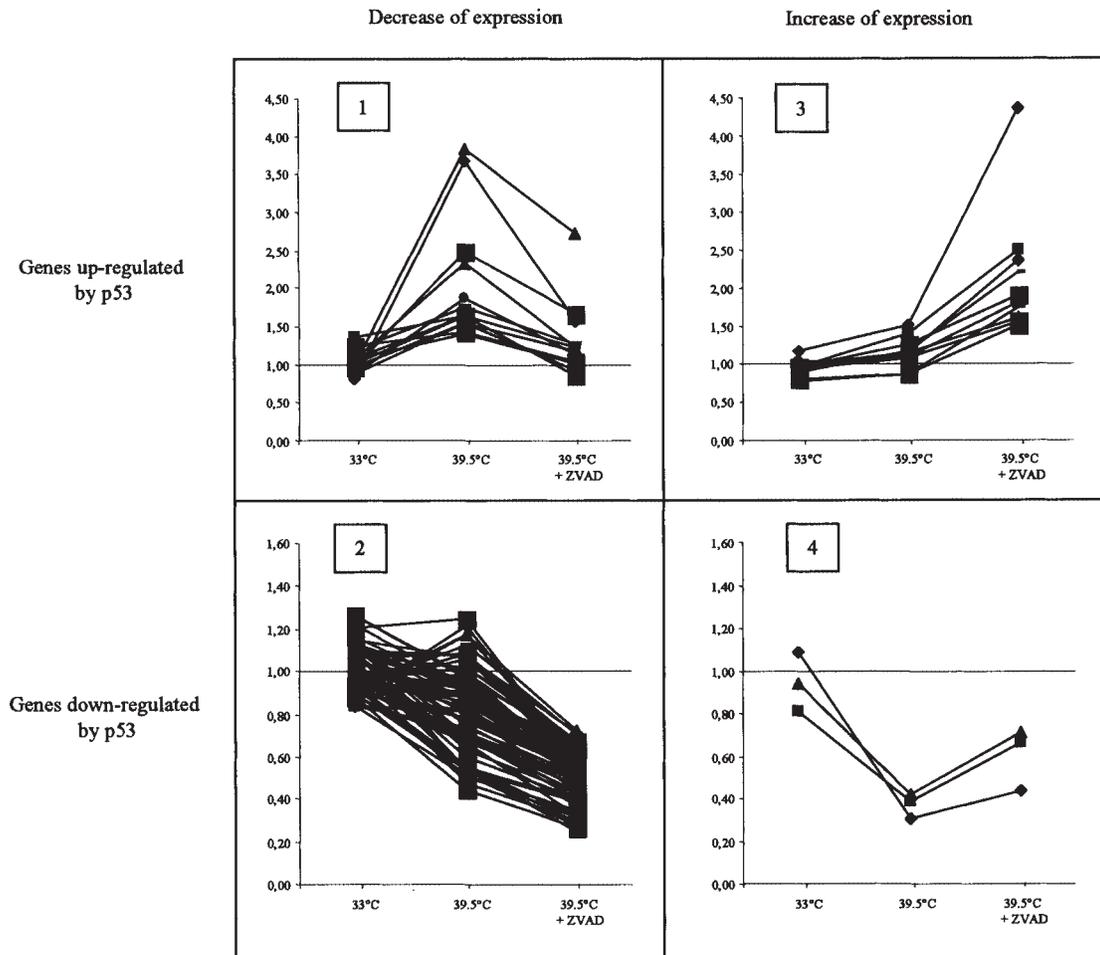


Figure 5. Cluster representation of genes differentially regulated by ZVAD addition. The 94 genes significantly regulated in the presence of ZVAD when p53 is active can be classified into four distinct clusters as listed in Table 2. Invariant expression (ratio = 1) is represented by the horizontal line.

Table 2. List of genes differentially regulated in the presence of ZVAD

Product	39/33	<i>P</i> -value	39+Z/33	<i>P</i> -value	Ratio (±)
Cluster 1					
<i>DF2</i>	3.66	0.00	1.57	0.15	0.43
ATP-binding cassette, sub-family A (ABC1), member 1	1.64	0.01	0.85	0.42	0.52
Gadd45	2.34	0.00	1.22	0.27	0.52
Inhibitor of DNA-binding 1	1.64	0.05	0.86	0.41	0.52
Chromobox homologue 5 (<i>Drosophila</i> HP1a)	1.55	0.01	0.92	0.23	0.59
Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	1.86	0.00	1.13	0.42	0.61
<i>SDM4E09</i>	1.44	0.03	1.03	0.11	0.72
Ubiquitin specific protease 4 (proto-oncogene)	1.41	0.01	1.01	0.89	0.72
<i>SDD3B10</i>	1.76	0.00	1.27	0.10	0.72
<i>RDM14F04</i>	1.63	0.00	1.22	0.13	0.74
Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	1.56	0.00	1.17	0.17	0.75
Serine (or cysteine) proteinase inhibitor, clade H, member 1	3.83	0.00	2.73	0.00	0.71
Insulin-like growth factor binding protein 4	2.48	0.00	1.65	0.02	0.66
Cluster 2					
Procollagen, type I, alpha 2	0.54	0.00	0.25	0.01	0.46
Dimethylarginine dimethylaminohydrolase 1	0.66	0.03	0.32	0.00	0.48
Procollagen, type III, alpha 1	0.52	0.00	0.27	0.01	0.53
Procollagen, type III, alpha 1	0.54	0.00	0.29	0.00	0.53
Procollagen, type III, alpha 1	0.56	0.00	0.32	0.01	0.56
Zinc finger protein of the cerebellum 1	0.65	0.01	0.37	0.00	0.57
Procollagen, type III, alpha 1	0.44	0.00	0.26	0.00	0.59
<i>GE1</i>	0.69	0.00	0.40	0.00	0.59
Myosin, heavy polypeptide 3	0.54	0.01	0.32	0.00	0.59
Procollagen, type III, alpha 1	0.51	0.01	0.31	0.01	0.61
Profilin 2	0.70	0.02	0.43	0.01	0.61
<i>RDM17A04</i>	0.48	0.00	0.30	0.00	0.62
Tropomyosin 1, alpha	0.68	0.00	0.44	0.00	0.64
<i>SDM3F12</i>	0.70	0.02	0.46	0.00	0.65
GM2 ganglioside activator protein	0.63	0.01	0.42	0.00	0.67
<i>SDD5G10</i>	0.60	0.01	0.41	0.00	0.68
ATP synthase, H ⁺ transporting, mitochondrial F1 cplx, alpha subunit, isoform 1	0.50	0.01	0.34	0.00	0.68
T-cell phenotype modifier 1	0.68	0.01	0.47	0.00	0.69
T-cell phenotype modifier 1	0.70	0.00	0.48	0.00	0.69
<i>SDM7G06</i>	0.71	0.01	0.50	0.00	0.71
Karyopherin alpha 1 (importin alpha 5)	0.55	0.00	0.40	0.00	0.72
<i>SDM4D07</i>	0.53	0.00	0.39	0.00	0.73
Thrombospondin 1	1.25	0.44	0.56	0.01	0.45
Agrin	1.21	0.43	0.55	0.01	0.46
<i>RDM2E06</i>	0.98	0.90	0.49	0.00	0.50
Annexin A1	1.17	0.36	0.60	0.02	0.51
JG1	1.07	0.65	0.55	0.00	0.52
Annexin A3	0.98	0.88	0.51	0.01	0.52
<i>t</i> -complex testis expressed 1	0.92	0.46	0.48	0.01	0.52
RNA polymerase II transcriptional coactivator	0.81	0.03	0.43	0.01	0.53
Growth arrest specific 6	1.09	0.08	0.59	0.02	0.54

Table 2. Continued

Product	39/33	<i>P</i> -value	39+Z/33	<i>P</i> -value	Ratio (±)
Integral membrane protein 2B	1.06	0.68	0.58	0.05	0.55
Cell division cycle 2 homologue A (<i>Saccharomyces pombe</i>)	0.92	0.19	0.51	0.00	0.56
Integrin beta 5	0.98	0.66	0.55	0.01	0.56
Polymerase (DNA directed), beta	0.97	0.78	0.54	0.01	0.56
Cell division cycle 2 homologue A (<i>S.pombe</i>)	1.08	0.44	0.63	0.00	0.58
Spectrin alpha 2	0.92	0.44	0.56	0.00	0.61
Calmodulin 2	0.95	0.48	0.58	0.00	0.61
High-mobility group nucleosomal binding domain 1	1.17	0.09	0.72	0.01	0.62
HLA-B associated transcript 2	0.86	0.18	0.53	0.01	0.62
Tubulin, alpha 1	0.87	0.33	0.54	0.02	0.62
<i>SDM5G10</i>	0.83	0.05	0.51	0.01	0.62
<i>RIKEN cDNA 2700016D05 gene</i>	1.07	0.64	0.67	0.01	0.62
Monoamine oxidase A	1.08	0.61	0.68	0.01	0.63
Proteasome (prosome, macropain) 28 subunit, 3	1.13	0.49	0.71	0.02	0.63
Malate dehydrogenase, soluble	0.92	0.43	0.58	0.01	0.64
Secreted acidic cysteine-rich glycoprotein	0.81	0.04	0.52	0.00	0.64
Pyruvate kinase, muscle	0.87	0.12	0.56	0.01	0.64
Integral membrane protein 2B <i>BF1</i>	1.02	0.84	0.66	0.00	0.64
Transcription factor E2a	0.75	0.07	0.48	0.01	0.64
<i>RDM10C12</i>	0.90	0.30	0.58	0.03	0.64
<i>RDM8B03</i>	0.99	0.96	0.64	0.00	0.65
<i>RDM10D02</i>	0.92	0.02	0.60	0.03	0.65
<i>RDM10D02</i>	0.80	0.04	0.52	0.02	0.65
Phosphoglycerate kinase 1	0.93	0.01	0.61	0.03	0.66
Methyltransferase-like 1	1.02	0.85	0.67	0.00	0.66
<i>AC2</i>	1.07	0.16	0.71	0.02	0.67
Tubulin, alpha 4	0.92	0.09	0.63	0.00	0.68
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	0.90	0.37	0.62	0.01	0.68
Tropomyosin 1, alpha	0.74	0.04	0.51	0.00	0.69
Inositol (myo)-1(or 4)- monophosphatase 1	0.96	0.61	0.67	0.00	0.70
Immunoglobulin joining chain	0.78	0.03	0.55	0.00	0.70
Cyclin G1	0.89	0.28	0.63	0.02	0.70
ADP-ribosylation factor 1	0.76	0.00	0.54	0.01	0.71
Adaptor protein complex AP-2, mu1	0.86	0.06	0.61	0.00	0.71
<i>RIKEN cDNA 2310075M17 gene</i>	0.84	0.09	0.60	0.00	0.72
Cyclin A2	0.88	0.09	0.63	0.01	0.72
Tubulin, alpha 8	0.90	0.36	0.66	0.02	0.73
Cluster 3					
Dual specificity phosphatase 1	1.52	0.04	4.37	0.00	2.88
Activating transcription factor 3	1.40	0.01	2.49	0.00	1.78
Cytochrome <i>b</i> , mitochondrial	1.14	0.28	1.61	0.01	1.42
<i>RDM6A12</i>	1.09	0.52	1.57	0.01	1.44
CD2	1.26	0.02	1.89	0.02	1.51
<i>RDM11C11</i>	1.09	0.46	1.82	0.02	1.67
High-mobility group AT-hook 1	0.86	0.43	1.52	0.03	1.76
XE2	1.18	0.10	2.21	0.01	1.88
<i>SDD2F12</i>	0.87	0.45	1.75	0.05	2.01
Myeloid differentiation primary response gene 116	1.15	0.03	2.37	0.00	2.05
Cluster 4					
Farnesyl diphosphate synthetase	0.30	0.00	0.43	0.00	1.45
Ring finger protein 14	0.39	0.00	0.66	0.04	1.71
Low-density lipoprotein receptor	0.42	0.00	0.71	0.00	1.69

The genes are grouped into four clusters according to the change in their expression after ZVAD addition. Their expression levels in the absence or presence of ZVAD with the corresponding *P*-values are listed in columns 1 and 2, respectively. The last column shows the ratio between these conditions. Unknown genes are in italics.

DISCUSSION

The tumour suppressor protein p53 induces cell cycle arrest or apoptosis, and is thus pivotal in suppressing cellular transformation and tumorigenesis. Cell cycle arrest is mediated by transcriptional induction of genes, the products of which inhibit proteins involved in cell cycle progression. The molecular events that lead to p53-dependent apoptosis are less clear. Transactivation of target genes may be involved but there is growing evidence that transrepression and transcription-independent functions are central to p53-dependent apoptosis (15,40). However, the physiological relevance of these apparently redundant pro-apoptotic properties and the determinism of choice between them are poorly understood. In the absence of caspase activity, p53 can induce a Bcl-2-insensitive cell death program (22,23). Here, we report that, in this Bcl-2-insensitive pathway unmasked by ZVAD, one or more ZVAD-sensitive caspases regulate p53 apoptotic functions by modulating activated biochemical properties of the protein.

Caspase inhibition modulates biochemical properties

The activation of the alternative pathway is associated with an increased stability, and accumulation, of p53 protein at restrictive temperature. This was expected to result in stronger induction of the p53-target genes, explaining the accelerating effect of ZVAD on p53-induced apoptosis. Surprisingly, we found that ZVAD-induced p53 accumulation is accompanied by a reduced transcriptional activation of its effectors. This discrepancy indicates both that the caspase inhibitor modulates p53 properties and that a transactivation-independent apoptotic function of p53 is required to trigger the alternative pathway.

Several studies are consistent with p53 promoting apoptosis through its transcriptional repression activity (12,15,36, 41–43): certain mutants or deleted forms of p53 that are unable to induce apoptosis have been found to be deficient in transrepression but not in transactivation (44,45); also, a positive association between p53-dependent repression and apoptosis has been demonstrated in some models (15,46).

Therefore, we evaluated the transrepression activity of p53 at restrictive temperature both in the presence and absence of the caspase inhibitor. We found that genes known to be repressed by p53 (*map4* and *stathmin*) were down-regulated by p53 activation both in the presence and absence of ZVAD. However, transrepression, which was only transient in the absence of ZVAD, was sustained and stronger in the presence of ZVAD, suggesting that its duration can be determined by the amount of p53.

We then conducted a large-scale study of the transcriptome. We showed that caspase inhibition leads to extensive changes in the pattern of regulated genes after p53 activation at restrictive temperature. The microarray analysis indicated that 86% (clusters 1 and 2) of the 94 genes modulated by ZVAD were more repressed or less activated in the presence of the drug; in other words, these genes are less expressed when caspases were inactive. Thus, the inhibition of caspases modifies the contribution of the different transcription activities of p53 to apoptosis—the onset of the Bcl-2-insensitive process is associated with both a decrease in transactivation and an increase in transrepression. Interestingly, none of the genes activated in the absence of ZVAD became repressed after addition of the drug and none of the repressed genes became activated in the

presence of ZVAD. This is consistent with p53-mediated transactivation and transrepression affecting different clusters of genes (as explained in Introduction).

Various different mechanisms have been described to account for p53-mediated repression. Inhibition of the NF-Y transcription factor (47,48) or up-regulation of *p21* which leads to hypophosphorylation of Rb and transcriptional repression via E2F1 binding (49), has been implicated. However, there is evidence that p53 can promote sequence-specific repression by binding proteins, like HDAC, that possess deacetylase and chromatin condensation properties. According to this model, our data suggest that the composition of transcriptional machinery complexes recruited by p53 on promoters might be modulated by a ZVAD-sensitive protease. Indeed, p53 can activate transcription through interaction with coactivators, e.g. p300 and CBP. Both of these proteins have histone acetylase (HAT) activity, which is critical for the transactivation function of p53. Conversely, to repress transcription p53 recruits histone deacetylases through a physical association with mSin3a. The time course of p53 association with mSin3a and p300 might differ according to the presence or absence of ZVAD. Interestingly, the C-terminal mSin3a-interaction domain and tetramerization domain of p53 overlap (43). Therefore, this interaction could contribute to the decrease in p53 transactivation in favour of increased transrepression. How ZVAD promotes the formation of this complex on promoters of p53-repressed genes remains unclear.

Nevertheless, the fact that a large number of genes are repressed in the presence of ZVAD suggests that these down-regulations may also be involved, at least in part, p53 indirectly controlling either the expression or the activity of other transcription factors.

Function of genes modulated by ZVAD

The most striking transcriptional modification associated with the commitment to the alternative apoptotic pathway is increased repression. We then focused on the function of genes that were down-regulated in the presence of ZVAD in order to elucidate the signalling of this new apoptotic pathway (see Figure 5 and Table 2).

Most of the genes affected were more repressed in the presence of ZVAD, and they included genes encoding components of the extracellular matrix and those of the cytoskeleton (*procollagen*, *myosin* and others). This is consistent with the caspase inhibitor accelerating the commitment to apoptosis. Indeed, we observed that morphological changes including the loss of adherence, cell shrinking and cell rounding were more rapid in the presence than in the absence of ZVAD. These events involve major alterations of both the cytoskeleton and the interactions between the cell and the extracellular matrix.

Numerous genes were not regulated by p53 in the absence of ZVAD but were repressed by p53 when caspases were inhibited. These genes are thus specifically implicated in the alternative pathway. They include genes encoding regulators of transcription, e.g. the *high mobility group nucleosomal binding domain 1* and the transcription factor *E2a*. Interestingly, *high mobility group nucleosomal binding domain 1* gene (*hmg1*), encodes an activator of transcription which binds to the minor groove of DNA, promoting transcription by inducing a conformational change of the chromatin (50). The *hmg 17*

gene, another member of the high-mobility group family, is a known primary target of p53 (51). Possibly *hmg1* is also a direct target of p53. The down-regulation of this type of transcription factor could trigger a process of amplification contributing to the overall transactivation decrease observed in the presence of ZVAD. Indeed, most of the genes identified in our analysis have not been reported to be p53 targets and our approach does not discriminate between genes directly repressed by p53 in a sequence-specific manner and genes repressed indirectly by a cascade of regulations. Another type of gene identified in our study was regulators of cell cycle (including *growth arrest specific 6*, *polymerase beta* and *cell division cycle 2 homolog A*). This is consistent with the established ability of p53 to arrest the cell cycle through transcriptional regulation. Furthermore, the commitment of REtsAF-Bcl-2 cells to apoptosis is associated with an inability to respond appropriately to p53-induced negative regulators of the cell cycle (52).

Surprisingly, 94 genes regulated by ZVAD included few genes known to be involved in apoptosis. There are several possible explanations: (i) many apoptotic genes were not represented on the array used, (ii) the signals given by these genes do not pass the statistical tests, (iii) the apoptotic genes were among the unidentified regulated genes and (iv) the Bcl-2- and caspase-insensitive pathway involves unidentified genes.

p53 protein level determines activated biochemical properties

Activation of the alternative pathway was associated with an increased stability of p53 at restrictive temperature. There was a parallel accumulation of MDM2, a consequence of a post-transcriptional mechanism. Presumably, these two proteins do not pass as rapidly to the proteasome as they do at restrictive temperature in the absence of ZVAD.

Control of p53 stability appears to be central in the determination of the p53 apoptotic pathway. Numerous studies show that the heterogeneous responses of gene transcription to p53 activation by diverse agents could be due to p53 protein levels differing according to the nature of the stress signal (37). In our model, target genes can be transactivated by p53 in a transient manner, the transient nature being determined by MDM2-mediated degradation of p53. In contrast, it could be argued that transcriptional repression is effective only if repression complexes are present on the promoters of repressed genes for prolonged periods (43,53). By protecting p53 from degradation, caspase inhibition may therefore enhance the effectiveness of p53 as a transrepressor. Note that two components of the proteasome were repressed in the presence of ZVAD (cluster 2 of DNA chip analysis), and this may contribute to the decrease in p53 and MDM2 proteasomal degradation in this condition.

However, the effect of caspase inhibition appears to be more puzzling. Unlike the effect on transrepression, p53 accumulation does not promote greater transactivation efficiency. As MDM2 also accumulates in the presence of ZVAD, probably as the result of inhibition of its proteasome-mediated degradation, it is possible that ZVAD blocks the caspase-mediated inactivation of a component which regulates, via MDM2, the stability and the apoptotic function of p53. Interestingly, Hsieh *et al.* (54) report that Rb, MDM2 and p53 can interact in a ternary complex in which Rb regulates the apoptotic function of p53.

The modification of the p53 protein itself depends on the types of stress (38): different types of DNA damage activate different protein kinases which phosphorylate different serine or threonine residues on the protein. The presence or absence of ZVAD may similarly cause different types of post-transcriptional modification. Whether these modifications qualitatively influence the outcome of p53 activation remains unclear. Thus, the overall transcription pattern of a cell might depend on the nature of qualitative and quantitative p53 status.

CONCLUSIONS

We report evidence supporting a new paradigm for the regulation of the apoptotic function of p53. We propose a new model in which one or more ZVAD-sensitive caspases determine the outcome of p53 activation by modulating the stability and biochemical properties of p53. When caspases are active, p53 activation is followed by a rapid MDM2-mediated degradation of p53 and a transactivation-dependent apoptotic program which engages the mitochondrial pathway (23). In contrast, in the absence of caspase activity, p53 activation triggers transrepression-dependent signalling for an alternative Bcl-2-insensitive (23) apoptotic program, which is associated with stabilization of p53 protein.

It is important to note that the effect of ZVAD is specific for p53 and not for LT antigen. Indeed, a dominant-negative form of p53 abolishes the proapoptotic action of ZVAD and ZVAD also potentiates p53-induced apoptosis in rat primary fibroblasts which do not express LT (23). This latter observation reinforces the idea that the caspase-dependent control of p53 activity has a physiological relevance. Nevertheless, this needs to be tested in human cells.

Further analysis and cataloguing of p53 targets genes regulated in response to various conditions (like ZVAD) may help to connect p53 activation to the apoptotic network. This could lead to the development of strategies to improve therapeutic treatment.

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