A novel p53-inducible gene, \textit{PAG608}, encodes a nuclear zinc finger protein whose overexpression promotes apoptosis

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The biological effects of the p53 tumor suppressor protein are elicited, at least in part, through sequence-specific transactivation of a battery of target genes. The differential display method was employed towards identifying additional p53 target genes, with emphasis on genes whose induction may contribute to p53-mediated apoptosis. We report here the cloning of a novel p53-inducible gene, designated \textit{PAG608}. \textit{PAG608} transcripts are induced by DNA damage in a p53-dependent manner. \textit{PAG608} encodes a nuclear zinc finger protein, which appears to localize preferentially to nucleoli when expressed at moderate levels in transfected cells. Transient overexpression of \textit{PAG608} in human tumor-derived cells leads to distinctive changes in nuclear morphology, and can promote apoptosis. Together with additional p53 target genes, \textit{PAG608} may therefore play a role in mediating the biological activities of p53.

Keywords: apoptosis/p53 tumor suppressor/\textit{PAG608}/zinc finger protein

Introduction

The p53 tumor suppressor gene is subject to frequent mutations in human cancer. A major outcome of such mutations is inactivation of the biochemical and biological functions of the wild-type (wt) p53 protein (for recent p53 reviews, see Bates and Vousden, 1996; Gottlieb and Oren, 1996; Ko and Prives, 1996; Oren and Prives, 1996). Among the biological effects elicited by wt p53, the best documented are cell cycle arrest and apoptosis.

The wt p53 protein can exert an array of biochemical activities. Of these, the most notable is its ability to act as a transcriptional regulator (Bates and Vousden, 1996; Gottlieb and Oren, 1996; Ko and Prives, 1996; Oren and Prives, 1996). p53 is a \textit{bona fide} positive transcription factor, capable of binding in a sequence-specific manner to well defined DNA elements and inducing the transcription of genes residing in the vicinity of such p53 response elements. In addition, p53 can repress the transcription of many other genes; here, however, direct sequence-specific interactions between p53 and these genes are generally not involved.

The cell cycle inhibitory activity of p53 is believed to rely primarily on its ability to act as a sequence-specific transcriptional activator. The gene whose contribution to p53-mediated growth arrest is best understood is the \textit{p21\textsuperscript{War1}} gene, encoding an inhibitor of cyclin-dependent kinases (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1996). Other genes, such as \textit{GADD45}, may also play a role in this response (Smith et al., 1994). Less is presently known about the biochemical basis of p53-mediated apoptosis. Depending on the particular experimental system, sequence-specific transactivation (SST) can either be obligatory (Sabbatini et al., 1995; Yonish-Rouach et al., 1995; Attardi et al., 1996; White, 1996) or dispensable (Caelles et al., 1994; Haupt et al., 1995b; Bates and Vousden, 1996) for apoptosis. It is most likely, though, that the optimal apoptotic effect of p53 requires a combination of both SST and other, SST-independent biochemical activities (Chen et al., 1996; Haupt et al., 1996; Wang et al., 1996).

Among the target genes whose sequence-specific transactivation by wt p53 may promote apoptosis, the most notable is \textit{bax} (Miyashita et al., 1994; Selvakumaran et al., 1994; Miyashita and Reed, 1995). Indeed, p53 mutants which retain the ability to activate numerous promoters, including that of \textit{p21\textsuperscript{War1}}, but carry a selective defect in activation of the \textit{bax} promoter, are competent for growth arrest but completely deficient for apoptosis (Friedlander et al., 1996; Ludwig et al., 1996). Other p53 target genes whose activation might contribute to apoptosis are \textit{IGFBP3} (Buckbinder et al., 1995; Friedlander et al., 1996; Ludwig et al., 1996) and the mammalian homolog of the \textit{Drosophila seven in absentia} gene (Amson et al., 1996; Nemani et al., 1996).

The list of presently identified p53 target genes is probably still far from exhaustive. To search for additional target genes we took advantage of the differential display method (Liang and Pardee, 1992; Zhao et al., 1996) already used successfully before for a similar purpose (Okamoto and Beach, 1994; Amson et al., 1996). In particular, the aim was to identify genes which might contribute to p53-mediated apoptosis. Therefore, the cell system chosen as a source of differentially expressed transcripts was one in which induction of wt p53 activity causes massive apoptosis. We report here the cloning of a novel p53 target gene, designated \textit{PAG608}. \textit{PAG608} encodes a nuclear zinc finger protein, whose overexpression promotes apoptosis in transfected human tumor-derived cells.

Results

\textit{PAG608} mRNA is induced by p53

In order to clone transcripts whose levels are modulated upon induction of biochemically active wt p53, we took
advantage of LTR6 cells. LTR6 cells are derived from the p53-deficient mouse myeloid leukemia line M1, stably transfected with the temperature-sensitive p53 mutant p53val135 (Yonish-Rouach et al., 1991, 1993). At 37.5°C, the p53val135 protein is predominantly in a mutant conformation, and does not exhibit biochemical and biological activities characteristic of wt p53. However, upon downshift to 32°C, it resumes a wild-type-like conformation, leading to induction of the corresponding biochemical and biological activities (Michalovitz et al., 1990). In the case of LTR6 cells, this results in massive p53-dependent apoptosis (Yonish-Rouach et al., 1991, 1993). RNA was extracted from LTR6 cells, either maintained at 37.5°C or incubated for 4 h at 32°C. The two RNA populations were subjected to screening by the differential display method (Liang and Pardee, 1992; Zhao et al., 1996); matched RNA samples from similarly treated parental, p53-null M1 cells served as controls for non-specific temperature effects. A non-exhaustive screen resulted in isolation of short PCR-amplified DNA fragments corresponding to six differentially expressed transcripts; three up-regulated and three others repressed in the presence of excess wt p53 activity (LTR6 cells, 32°C). Of the former, one was found to represent a member of the GLN long terminal repeat (LTR) family of mouse endogenous retrovirus-like elements (data not shown), already identified earlier as a target for direct transcriptional activation by p53 (Zauberman et al., 1993). Sequencing of the other two p53-induced, short PCR-amplified DNA fragments suggested that they represent novel genes. Of these two, clone PAG608 (p53-activated gene 608) displayed stronger and more consistent induction by p53, when assessed by semi-quantitative RT-PCR using RNA from LTR6 cells and from several other cell systems in which wt p53 activity can be elevated experimentally (see below, and data not shown). PAG608 was therefore chosen for further study.

In order to confirm the differential display and RT-PCR data, Northern blot analysis was performed for PAG608 mRNA. RNA from LTR6 cells was subjected to agarose gel electrophoresis, transferred onto a nylon membrane and probed with a PAG608 cDNA probe. The radiolabeled probe consisted of the most 5' 2.5 kb of PAG608 cDNA, including the entire open reading frame (see below). The membrane was subsequently re-probed for GAPDH. The positions of size markers and 18S and 28S rRNA are indicated.

shift (Figure 2A). To assess the approximate fold induction of PAG608 mRNA, semi-quantitative RT-PCR was performed on serial dilutions of cDNA from LTR6 cells maintained for 4 h at 32°C. As seen in Figure 2B, comparison with undiluted cDNA from 37.5°C cells indicated a 10- to 20-fold increase at 32°C relative to the non-permissive temperature.

The fast induction kinetics of PAG608 mRNA suggested strongly that the corresponding gene is a direct target of wt p53, rather than being induced as a secondary consequence of p53 activation. To support this conclusion further, PAG608 induction was examined in the presence of the protein synthesis inhibitor cycloheximide. As seen in Figure 2C, cycloheximide did not interfere significantly with the p53-dependent increase in PAG608 mRNA. The cycloheximide treatment inhibited protein synthesis by >90%, as determined by measuring [35S]methionine incorporation (data not shown). Hence, de novo protein synthesis is not required for induction of PAG608 gene expression by p53. This provides additional evidence that PAG608 expression is regulated directly by wt p53.

PAG608 mRNA is induced by DNA damage in a p53-dependent manner

In both LTR6 and C16 cells, the temperature shift results in a vast, presumably non-physiological surge of wt p53 activity. To confirm that PAG608 expression can be induced
Fig. 3. Induction of PAG608 mRNA by DNA damage. (A) p53-deficient (p53+/–) mice (Lowe et al., 1993), as well as wild-type (WT) littermates, were obtained through a cross between a male and female p53+/+ mouse. One 40-day-old mouse of each type was exposed to 5 Gy of whole-body γ-irradiation (+ IR), and another pair was left unirradiated (– IR). Four hours later, total RNA was extracted from the thymus of each mouse, and 8 μg of RNA was taken for semi-quantitative RT–PCR with PAG608- and mouse GAPDH-specific primers. The number of amplification cycles was 27 or 23 for PAG608 and GAPDH, respectively. (B) RNA was extracted from mouse myeloid 32D cells, either untreated (–) or exposed for 24 h to 1 μg/ml cisplatin (+). Semi-quantitative RT–PCR was performed as above. The number of amplification cycles was 23 or 19 for PAG608 and GAPDH, respectively.

Fig. 4. Tissue distribution of PAG608 mRNA. Total RNA was extracted from various tissues of an adult rat. Equal amounts (10 μg) of RNA from each tissue were subjected to 30 cycles of RT–PCR with PAG608-specific primers; the same cDNAs were subjected in parallel to 25 cycles of PCR with GAPDH-specific primers. B, brain; G, gut; H, heart; K, kidney; LI, liver; LU, lung; M, skeletal muscle; S, spleen; T, thymus.

by activation of endogenous wt p53 under more physiological conditions, semi-quantitative RT–PCR was performed with RNA extracted from the thymuses of wild-type mice and p53 knock-out (p53+/–) mice, either untreated or 4 h after exposure to whole-body ionizing radiation. As seen in Figure 3A, a several fold increase in PAG608 mRNA was observed upon irradiation of normal mice, but not of p53+/– mice. Hence induction of DNA damage in the thymus, known to trigger vigorous p53-dependent apoptosis (Clarke et al., 1993; Lowe et al., 1993), also stimulates PAG608 expression in a p53-dependent manner.

Induction of PAG608 was also assayed in mouse myeloid 32D cells. 32D cells contain wt p53, which can be activated by DNA damage (Hiebert et al., 1995). Treatment of these cells with cisplatin increases the expression of p21Waf1, a prototype p53 target gene (G.Blandino and M.Oren, unpublished). In parallel, PAG608 expression is also induced (Figure 3B). Thus, PAG608 indeed behaves as expected of a proper p53 target gene.

To determine the in vivo expression pattern of PAG608 transcripts, RNA was extracted from several rat tissues and subjected to RT–PCR. Expression could be detected in all tissues examined: the highest levels were encountered in brain, gut, lung and testis (Figure 4).
PAG608, a novel p53-inducible gene

Fig. 6. Detection of endogenous and transfection-derived PAG608 protein. LTR6 (lanes 3 and 4), Cl6 (lanes 5 and 6) and DP16val135 (lanes 7 and 8) cells were maintained at either 32 or 37.5°C, as indicated above each lane. Total cell extracts were subjected to Western blot analysis using a polyclonal rabbit serum raised against a GST fusion protein containing the last 59 residues of the rat PAG608 ORF. Lanes 1 and 9 contain extracts from p53-null human H1299 cells, transiently transfected with an expression plasmid (pHA-PAG608) encoding the full-length PAG608 ORF fused at its N-terminus to a HA tag. Lane 2 contains an extract of mock-transfected H1299 cells.

The presence of an in-frame translation termination codon within the 5' UTR (see Figure 5) further supports the correct assignment of the first methionine of the ORF.

This ORF predicts a protein of 289 amino acid residues, containing three conserved zinc finger motifs of the C2H2 type (Figure 5, underlined). No other distinctive motifs could be discerned.

The 7.5 kb RNA sequence most probably corresponds to the large rat PAG608 transcript (Figure 1). Analysis of several partial cDNA clones, as well as PCR amplification of DNA from the cDNA library with several pairs of primers, did not provide evidence for alternative splicing (data not shown). The shorter transcripts shown in Figure 1 may thus represent products of alternative polyadenylation. In fact, analysis of the complete 7.5 kb cDNA shown are the first 1090 nucleotides of rat PAG608 cDNA, comprising sequence reveals potential polyadenylation signals at ~2.3 kb and 6.4 kb downstream from the transcriptional start site, and as well as the last 100 nucleotides of the mRNA. No conspicuous open reading frame (ORF) could be identified (data not shown).

Expression of the PAG608 protein is induced in a p53-dependent manner

A polyclonal rabbit serum was raised against a GST fusion protein containing the last 59 residues of rat PAG608. Using this antiserum, strong induction of a polypeptide corresponding to the extreme 3' end of the mRNA was observed at 32°C in LTR6, Cl6 and DP16val135 cells (Figure 6). The latter cells, derived by stable transfection of the mouse erythroleukemia cell line with p53 val135, also undergo apoptosis when wt p53 activity is induced at 32°C (Johnson et al., 1993). The antiserum also detected a corresponding polypeptide in human p53-null H1299 cells (data not shown). All corresponding transcripts are therefore expected to vary only at their 3' ends, and encode the same 289 amino acid protein.

As a first step towards determining the full sequence of PAG608 mRNA, the short differential display cDNA fragment, corresponding to the extreme 3' end of the mRNA, was employed for screening an LTR6 (32°C) cDNA library. This resulted in isolation of a total of 7.5 kb. This extends from the 3' end of the mRNA to very close to the transcriptional start site, as determined by Marathon RACE (data not shown). Overall, the sequence reveals a rather short 5' -untranslated region (5' UTR), followed by a relatively short ORF and a very long 3' UTR (Figure 5; only part of the 3' UTR is shown).

The presence of an in-frame translation termination codon within the 5' UTR further supports the correct assignment of the first methionine of the ORF.
the PAG608 protein also accumulates in the presence of activated wt p53.

**PAG608 is a nuclear protein**

To determine the subcellular localization of the PAG608 protein, human Saos-2 osteosarcoma cells were transiently transfected with a PAG608 expression plasmid. Forty-eight hours later, cells were fixed and stained with the PAG608 antiserum; nuclear DNA was visualized in the same cells by simultaneous 4',6-diamidino-2-phenylindole (DAPI) staining. In the majority of successfully transfected cells, the protein localized exclusively to the nucleus (Figure 7A and B). In a small percentage of cells, staining of variable intensity could also be observed in the cytoplasm (data not shown). The precise pattern of staining within the nucleus varied with the extent of PAG608 overexpression. In cells with relatively limited expression, the protein was localized primarily to a number of small regions within the nucleus, most likely representing nucleoli. This is exemplified by the cell at the bottom of Figure 7C; the corresponding DAPI staining is seen in Figure 7D, and a combined DAPI + phase image in Figure 7E. Cells with higher amounts of PAG608 protein displayed a more widespread nuclear staining. Examination of such cells at higher magnification revealed that in some of them the PAG608 protein was not uniformly distributed throughout the nucleus, but rather exhibited a heavily punctate pattern (Figure 7F). Remarkably, multiple speckles of condensed chromatin were evident within the corresponding nuclei (Figure 7G, and some additional examples in B and I); often, these speckles were most intense at positions from which PAG608 protein was excluded (compare Figure 7F with G and H with I). It is noteworthy that a similar pattern, i.e. nucleolar staining at lower expression levels and wider nuclear staining at higher levels, has been reported for another zinc finger protein, LYAR (Su et al., 1993).

**Overexpression of PAG608 induces apoptosis**

A transient transfection assay (Haupt et al., 1995a) was employed in order to gain insight into the cellular consequences of PAG608 overexpression. To that end, Saos-2 and human lung adenocarcinoma H1299 cells were transiently transfected with a PAG608 expression plasmid. Cultures were harvested 48 (Saos-2) or 66 h (H1299) later, fixed and stained for PAG608. Subsequently, the DNA of the same cells was stained with propidium iodide (PI). Appropriate gating in the FACS was then used to separate the successfully transfected cells, expressing high levels of PAG608, from the bulk of non-transfected cells (Figure 8A; the bar represents the boundary between the two sub-populations). The gated only, PAG608-overexpressing sub-population is shown in Figure 8B. Analysis of DNA content revealed that this gated sub-population exhibited a significantly higher fraction of cells with sub-G1 DNA content, indicative of apoptosis (Darzynkiewicz, 1995). This was observed for both H1299 cells (compare Figure 8D with C) and Saos-2 cells (compare Figure 8F with E). A two-parameter analysis (Figure 8K and L) revealed that many of the sub-G1 cells were of a size similar to or only slightly smaller than the rest of the cells. Hence, these sub-G1 cells most probably represent true apoptotic cells rather than random small cellular debris. In addition, in H1299 but not Saos-2 cells, there was also a reproducible increase in the fraction of cells in G2/M, the nature of which is presently unclear. The combined data from three independent transfection experiments is presented in Figure 9; the differential effect of PAG608 overexpression is less impressive in Saos-2 cells, owing to a variably high background of spontaneous apoptosis in cultures of this cell line.

For controls, Saos-2 cultures were transfected in parallel with expression plasmids for different forms of p53. These
cultures were then analyzed as above, except that a p53-specific monoclonal antibody was used for gating (Haupt et al., 1995a). Overexpression of wt p53 elicited substantial apoptosis in Saos-2 cells (compare Figure 8H with G), in line with earlier reports (Yonish-Rouach et al., 1995; Haupt and Oren, 1996; Rowan et al., 1996). By contrast, no such effect was exerted by mutant p53dl(13–52) (Figure 8I and J), which contains a deletion within the transactivation domain and is deficient in the induction of apoptosis (Haupt et al., 1995b).

Many cells within the PAG608-positive sub-population displayed a variety of nuclear aberrations characteristic of apoptosis (Figure 7). These included deformed or fragmented nuclei, regions of intense DAPI staining, cytoplasmic shrinkage (arrowhead, Figure 7J) and ‘ghosts’, where hardly any chromosomal DNA was left (arrows, Figure 7H and J). Moreover, cells displaying heavy speckles of condensed chromatin also stained positive by TUNEL (Figure 10). Thus, high levels of PAG608 protein can promote apoptosis in at least two distinct cell lines.

**Discussion**

In the present study, we report the cloning and characterization of a novel gene, designated PAG608, whose expression is strongly activated by wt p53. When overexpressed in transfected cells, the relatively small zinc finger protein encoded by PAG608 exhibits a predominantly nuclear localization, although it does not contain a canonical nuclear localization signal. Moreover, the staining pattern of the transfected protein suggests that it is localized preferentially to nucleoli. So far, however, we have been unable to obtain unequivocal staining of the endogenous PAG608 protein in non-transfected cells, suggesting that it is present in rather low amounts. The nucleolar localization of the PAG608 protein is compatible with a possible role in regulation of rRNA synthesis. Interestingly, the pRb protein, product of another tumor suppressor gene, has been shown to accumulate in nucleoli and block the transcriptional activity of RNA polymerase I, and this has been suggested as an alternative anti-proliferative mechanism (Cavanaugh et al., 1995). It is tempting to speculate that PAG608, too, may mediate particular inhibitory effects through interference with optimal production of new ribosomes.

The biological significance of p53-mediated PAG608 induction remains unknown, as is also largely still the case for some of the better-known p53 target genes. Nevertheless, the conspicuous induction of PAG608 in cells undergoing p53-dependent apoptosis, and particularly its ability to promote apoptosis in transiently transfected cells, raise the possibility that it plays some role in facilitating the apoptotic activities of p53. It should be borne in mind that, unlike what one observes upon extensive overexpression of PAG608, it seems rather unlikely that physiological induction of any single p53 target protein will suffice by itself to trigger apoptosis efficiently. Under physiological conditions, p53-mediated induction of genes that promote cell cycle arrest and programmed cell death may be required.

![Fig. 8. Induction of apoptosis in H1299 and Saos-2 cells by transient overexpression of PAG608.](Image)

Cells plated at a density of 10^6 per 10 cm dish were transfected with expression plasmids encoding the following proteins under control of the CMV early enhancer/promoter: PAG608 (A–F, K and L), wild-type mouse p53 (G and H) or the mouse p53 deletion mutant p53dl(13–52) (I and J). Forty eight (Saos-2) or 66 h (H1299) later, adherent and floating cells were combined, fixed with methanol, stained with antibodies specific for the corresponding transfection-derived protein and with propidium iodide (to visualize DNA content), and analyzed by flow cytometry. The polyclonal PAG608 antiserum was used for (A–F), and (K) and (L), whereas the p53-specific monoclonal antibody PAb421 was employed for (G–J). To differentiate the successfully transfected cells from the bulk non-transfected population in each culture, specific protein staining was plotted as a function of cell size (forward scatter, illustrated in A for PAG608-transfected Saos-2 cells). PAG608 fluorescence is plotted on a logarithmic scale. Small cellular debris was eliminated by leaving out all particles whose size (forward scatter) was substantially below that of the cells in the bulk population. A gate (denoted by bar in A) was then set, and cells with high PAG608 staining (above the bar) were monitored separately. The size and fluorescence distribution of the high-PAG608 cells are shown in (B). The DNA content distributions of the total (A) and gated (B) populations are depicted in (E) and (F), respectively. A similar gating and analysis protocol was also employed for PAG608 in H1299 cells (C and D), as well as for p53 in G–J. The horizontal bar in (C–J) denotes the position of cells with sub-G1 DNA content, indicative of apoptosis; the percentage of sub-G1 cells within the total population is indicated above the bar. A two-parameter presentation of the same cells as shown in (C) and (D), where DNA content is plotted as a function of cell size for the total (K) and the PAG608-positive (L) populations.
apoptosis appears to be elicited through the combined effect of several distinct pathways; only some of these pathways rely on the ability of p53 to act as a sequence-specific transcriptional activator of target genes (Haffner and Oren, 1995; Bates and Vousden, 1996; Gottlieb and Oren, 1996). Moreover, despite the centrality of bax in that latter group of target genes (Miyashita et al., 1994; Selvakumaran et al., 1994; Miyashita and Reed, 1995; White, 1996), it is most probable that bax is only one of several genes whose activation by p53 contributes to apoptosis. Our data suggest that PAG608 may be another member of this group, perhaps acting through a mechanism totally unrelated to that of bax. Recent studies suggest that the transcriptional regulation of apoptosis-mediating genes by p53 may be uncoupled, under certain circumstances, from that of growth arrest-mediating genes (Bates and Vousden, 1996; Friedlander et al., 1996; Ludwig et al., 1996). It will therefore be of interest to find out whether PAG608 gene expression is also subject to similar differential regulation.

Further insight into the biological role of PAG608 is likely to be gained once its biochemical functions are better understood. For the time being, the main clue is the presence of the three zinc fingers. This feature might suggest binding to nucleic acids, although an involvement in specific protein–protein interactions cannot be ruled out either. It is of note that several p53 target genes encode zinc finger proteins; these include mdm2 (Barak et al., 1993; Wu et al., 1993), HIC-1, a putative tumor suppressor gene (Wales et al., 1995), the mammalian homolog of the Drosophila seven in absentia gene (Amson et al., 1996; Nemani et al., 1996), and now PAG608. In addition, the p53 protein forms physical associations with several zinc finger proteins, including WT1 (Maheswaran et al., 1995) and also Mdm2 (Momand et al., 1992; Oliner et al., 1992; Barak et al., 1993). Thus, nuclear processes involving the activity of zinc finger proteins appear to represent a favorite target for modulation by p53. Future work on PAG608 will hopefully allow better elucidation of the nature of these processes.

Materials and methods

Cell lines

M1 and LTR6 cells were maintained routinely at 37.5°C in RPMI supplemented with 10% fetal calf serum (FCS). 32D cells were maintained in RPMI supplemented with 10% FCS and 0.1% medium conditioned by X63/0 cells (Karasuyama and Melchers, 1988) serving as a source of interleukin-3. C16, Saos-2 and DP16val135 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS.

Differential display cDNA cloning

Differential display cDNA cloning of p53-inducible transcripts, using RNA from M1 and LTR6 cells maintained either at 32 or at 37.5°C, was performed essentially as described before (Amson et al., 1996). In the present study, three 3′ primers (out of a total of four) were used in combination with four 5′ primers (out of a total of 12) and only part of the differentially displayed bands were taken for further analysis. Thus, the screening was non-exhaustive.

Semi-quantitative RT–PCR

Total RNA was extracted by the RNAzol™ (TEL-TEST, Inc.) procedure. First-strand cDNA was synthesized with M-MLV reverse transcriptase (Promega) in the presence of 10 μg of RNA, 0.25 μg of oligo(dT) primer, 0.5 mM each of dATP, dCTP, dGTP and dTTP, 25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 1.5 mM MgCl2, 5 mM dithiothreitol (DTT) and 200 U of M-MLV reverse transcriptase, in a final volume of 20 μl. For each PCR reaction, we used 1 μl of cDNA, 0.1 mM each of dATP, dCTP, dGTP and dTTP, 2 U of Taq polymerase (Advanced Biotechnologies), 1× of the corresponding reaction buffer (provided with the enzyme) and a pair of primers specific for either PAG608 or GAPDH (0.75 μM each).

The following primer combinations were used: PAG608, 5′ CTTTGGTTTTATTTTCTTCAGCC3′ and 5′ ACAGCGGAGATAGCCCAACGTAA3′; GAPDH, 5′ CAGCAATGCTCAGCACC3′ and 5′ TGGACTGTGGTCATGAGCCC3′. A limiting cycle amplification protocol was used in order to maintain the PCR reaction within its linear range. To determine the optimal number of amplification cycles for each pair of primers, aliquots of the corresponding PCR reactions were withdrawn for agarose gel analysis after a minimal number of cycles. The reaction was then resumed for two additional cycles, and aliquots were withdrawn at various cycles.

Fig. 9. Induction of apoptosis by PAG608 overexpression. Graphical representation of the flow cytometric analysis of PAG608-transfected cultures. Experimental details are as described in Figure 8. Each column represents at least three independent transfection experiments. Standard deviation is indicated by vertical bars.

Fig. 10. PAG608-induced nuclear alterations correlate with positive TUNEL staining. Saos-2 cells were transiently transfected with PAG608 as described for Figure 7, fixed in paraformaldehyde and subjected simultaneously to TUNEL analysis (A) and DAPI staining (B).
again. This procedure was repeated several more times, with an increasing number of cycles. The presented data were obtained with the minimal number of cycles sufficient for detecting clearly visible PCR bands.

**Northern blot analysis**

Northern blot was performed as described before (Barak et al., 1993), using a PAG608 cDNA probe or a GAPDH control probe.

**DNA sequencing**

DNA sequencing was performed by the Weizmann Institute DNA sequencing unit, with the aid of an ABI 373 automatic sequencer. Sequence analysis was carried out with the DNA sequence assembly software AutoAssembler™ (ABI).

**Production of PAG608-specific antisemur**

The last 59 residues of the PAG608 ORF were fused in-frame with GST, in the pGEX-3 plasmid (Pharmacia). The fusion protein was overexpressed in bacteria and purified as described (Barak et al., 1993), and then used for immunization of New Zealand White rabbits.

**Protein analysis**

Total cell extracts were analyzed by Western blotting as described (Barak et al., 1993), using the rabbit PAG608 antisemur followed by protein A-conjugated horseradish peroxidase. The blots were developed with the ECL chemiluminescence system (Amersham).

**Flow cytometry**

H1299 or Saos-2 cells were plated 24 h before transfection at a density of 1.2×10^6 cells per 10 cm dish. Cells were transfected by the calcium phosphate co-precipitation method, as described before (Haupt et al., 1995). The precipitate was left on the cells for 4–6 h (H1299) or overnight (H1299). Amounts of transfected plasmid DNA per 10 cm dish were 3 and 10 μg for p53 and PAG608, respectively. At 48 (H1299) or 66 h (H1299) post-transfection, adherent and floating cells were collected together and fixed in methanol. Fixed cells were resuspended and incubated in phosphate-buffered saline (PBS) at 4°C for at least 10 min. Subsequently, the cells were reacted with the primary antibody (PAG608-specific antisemur or the p53-specific monoclonal antibody PAb421, as appropriate). PI (Sigma) was added to the cell suspension to a final concentration of 50 μg/ml, and cells were analyzed in a cell sorter (FACSORT, Becton Dickinson). Cells were first monitored for overexpression of the transfected gene product, by determining their fluorescein isothiocyanate (FITC) fluorescence intensity. A threshold (‘gate’) defining the high FITC sub-population was set empirically (see Results). Equal numbers of cells from the total population and from the gated, protein-overexpressing sub-population were recorded separately for PI staining.

**Immunofluorescence microscopy**

Saos-2 cells were plated on glass cover slips within 6 cm tissue culture dishes. Transfections were as described above. Cells were washed in ice-cold PBS, fixed in cold methanol (−20°C) for at least 20 min and rehydrated in ice-cold PBS for at least 30 min. Following incubation with rabbit anti-PAG608 serum for 60 min at room temperature, cover slips were washed three times with PBS and incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. The last incubation contained DAPI (Sigma) at a concentration of 0.5 μg/ml. Cover slips were washed three times in PBS, mounted over glass slides and viewed under a fluorescence microscope (Zeiss Axioscope).

**TUNEL analysis**

Cells were plated on coverslips, transfected and harvested as described above, fixed in 3% paraformaldehyde for 30 min at room temperature and washed in PBS. The fixed coverslips were rinsed and incubated in the reaction buffer provided by the supplier (Boehringer), supplemented with 1.5 mM CoCl2, 50 μM biotin-16-DUTP (Boehringer) and 10 μM of terminal deoxynucleotidyl transferase (Boehringer) for 1 h at 37°C. Coverslips were then washed for 15 min in 20× SSC, rinsed twice in PBS, and incubated for 30 min at 37°C with PBS containing ExtraAvidin-TRITC, and DAPI at 0.5 μg/ml. Following two washes in PBS, the coverslips were mounted over glass slides and viewed under the fluorescence microscope.

**Accession number**

The DDBJ/EMBL/GenBank accession number for the PAG608 sequence is Y13148.

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D.Israeli et al.


