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# Isolation of 10 differentially expressed cDNAs in p53-induced apoptosis: Activation of the vertebrate homologue of the *Drosophila* seven in absentia gene

(tumor suppression/development/MEN1/ZFM1/PLC)

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**ABSTRACT** **We report the isolation of 10 differentially expressed cDNAs in the process of apoptosis induced by the p53 tumor suppressor. As a global analytical method, we performed a differential display of mRNA between mouse M1 myeloid leukemia cells and derived clone LTR6 cells, which contain a stably transfected temperature-sensitive mutant of p53. At 32°C wild-type p53 function is activated in LTR6 cells, resulting in programmed cell death. Eight genes are activated (TSAP; tumor suppressor activated pathway), and two are inhibited (TSIP, tumor suppressor inhibited pathway) in their expression. None of the 10 sequences has hitherto been recognized as part of the p53 signaling pathway. Three TSAPs are homologous to known genes. TSAP1 corresponds to phospholipase C β4. TSAP2 has a conserved domain homologous to a multiple endocrine neoplasia I (ZFM1) candidate gene. TSAP3 is the mouse homologue of the *Drosophila* seven in absentia gene. These data provide novel molecules involved in the pathway of wild-type p53 activation. They establish a functional link between a homologue of a conserved developmental *Drosophila* gene and signal transduction in tumor suppression leading to programmed cell death.**

A global view of the molecular events occurring during the cell cycle, development, and apoptosis, is necessary in order to understand the basis of cancer and tumor suppression. An overall and systematic analysis of gene expression modulation, responsible for tumor suppression, was hitherto hampered by two factors. A technological restriction, which has recently been overcome with the description of differential cDNA display (1), procuring the possibility to screen and compare expression of the majority of genes. The second restriction lies in the choice of the model system to be analyzed. The biological models must be well established, providing for a functional link, and suitable for differential molecular comparison. Such studies should lead beyond isolation of single tumor suppressor genes to definition of the entire molecular pathways of apoptosis and suppression. In human tumors, the most frequently mutated gene is that for p53 (2, 3). Two main streams of evidence indicate that p53 is a tumor suppressor gene. First, transfection experiments demonstrate that wild-type p53 induces growth arrest or apoptosis (4–7). Second, nullizygous mice for the p53 gene are significantly more susceptible to tumor formation (8). The molecular pathways to achieve p53 tumor suppression remain unclear, and hitherto several genes were characterized, which are induced by p53 (9–16). To further isolate genes activated by p53, we have performed comparative cDNA display analysis on M1 myeloid cells as well as on LTR6 cells. The latter are a subclone of the M1 cells stably transfected with a temperature-sensitive mutant p53 gene (7). At 37°C, Val-135 p53 (6) has a conformation and function similar to that of typical tumor-derived mutants. At 32°C, it functions like wild-type p53 and induces in M1 cells the program of cell death (7).

The present work describes identification of 10 differentially expressed cDNAs during the first hours of apoptosis induction.** TSAP1 and TSAP2 are homologous to two human genes localized in the multiple endocrine neoplasia 1 susceptibility region on chromosome 11q13 (17–19). Our data suggest that both phospholipase C β4 (TSAP1) and ZFM1 (TSAP2) are involved in the p53 signal transduction pathway. Most interestingly, one of the genes activated by wild-type p53 is the vertebrate homologue of the *Drosophila* seven in absentia gene (20, 21). In Drosophila, seven in absentia codes for a metal binding nuclear protein, downstream of the sevenless receptor. This gene is required for specification of the R7 photoreceptor cells in *Drosophila* eye development (20). The vertebrate homologue (*Siah*) is activated during development in various tissues, among which are forebrain and germ cells (21, 22). Activation of *Siah* suggests that pathways involved in developmental signaling may also participate in mediating the apoptotic activity of p53.

## MATERIALS AND METHODS

**Cell Cultures.** M1 myeloid leukemia cells (clone S6) and M1 cells stably transfected with the mutant temperature-sensitive Val-135 p53 (LTR6) were cultured in RPMI 1640 medium with 10% fetal calf serum in 5% $CO_2$/95% air at 37°C (7). For the temperature shift, cultures were placed in a second incubator

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Abbreviations: TSAP, tumor suppressor activated pathway; TSIP, tumor suppressor inhibited pathway; LTR, long terminal repeat.
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**The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U50959 (TSAP1), U50960 (TSAP2), U50958 (TSAP3), U50953 (TSAP4), U50956 (TSAP5), U50961 (TSAP6), U50954 (TSAP7), U50955 (TSAP8), U50962 (TSIP1), U50957 (TSIP2)].

at 32°C. For all assays performed in this study, cells were checked after 12 and 24 hr for the presence of apoptosis.

**Modified Differential cDNA Display.** To perform the assays in standard experimental conditions and to obtain full reproducibility of results, the following changes to the original protocol (1) were made. We always used poly(A)$^+$ mRNA purified twice on an oligo(dT) column using Fast Track (Invitrogen). After the reverse transcription (M-MLV reverse transcriptase; GIBCO/BRL) reaction on 0.05 $\mu$g of poly(A)$^+$ using 20 $\mu$M of each dNTP (Boehringer Mannheim), no additional dNTPs were added to the final PCR (GeneAmp; Perkin–Elmer) mixture. A hot start at 94°C for 5 min was performed before the PCR (GeneAmp PCR system 9600 Perkin–Elmer/Cetus) and the samples were snap cooled on iced water. A Touch Down (23) of 10 cycles from 50°C to 40°C was performed (94°C for 30 sec, 50°C for 1 min, 72°C for 30 sec), followed by 35 cycles (94°C for 30 sec, 40°C for 1 min, 72°C for 30 sec), and a final extension of 5 min at 72°C. The PCR products were separated on nondenaturing 6% polyacrylamide gels (24). Gels were exposed without drying. Each differential display was performed by comparing M1S6 and LTR6 at 37°C and after 4 hr of incubation of both cell lines at 32°C. All the differential display procedures were repeated in three separate experiments to confirm full reproducibility.

Differentially expressed bands were cut out of the gel, eluted, and reamplified (1). PCR products were subcloned using the TA-cloning system (Invitrogen), following the manufacturer's instructions. For each ligation reaction, 10 recombinant clones were sequenced using the automated ABI system.

**RNA Extraction, Northern Blot Analysis, and Probes.** Total RNA was extracted with Trizol (GIBCO/BRL Life Technologies, Gaithersburg, MD). Poly(A)$^+$ RNA was prepared using the OligotexdT kit (Qiagen, Chatsworth, CA). Thirty micrograms of total RNA or 2 $\mu$g of poly(A)$^+$ RNA was separated on a 1% agarose/1× Mops/2% formaldehyde gel and transferred to nylon membranes (positive membrane; Appligene, Strasbourg, France) as described (25). Northern blots were hybridized with random-primed $^{32}$P-labeled probes corresponding to TSAP and TSIP inserts and washed as described (25). To verify induction of wild-type p53 function, Northern blots were hybridized with a cyclin G probe (16). As control for the amount of mRNA loaded, blots were hybridized with a GAPDH probe. Multiple tissue Northern blots (Clontech) were used under identical conditions and hybridized for the control with a $\beta$-actin probe. The reverse transcription PCR product of LTR6 was amplified using *siah1b* primers 5′-CAGTAAACCACTGAAAAACC-3′ and 5′-CAAACCAAACCAAAACCAC-3′. The subcloned PCR product was used as the *siah1b* control probe. Northern blots were exposed for 2–10 days at −80°C.

**Slot Blots.** Slot blots were used to confirm the data reproducibly obtained by Northern blot analysis. Blots were prepared (Bio-Rad) by spotting PCR products (200 ng on Zeta-Probe Blotting Membranes, Bio-Rad, following the instructions of the manufacturer) of TSAP clones and hybridized to a total $^{32}$P-labeled random-primed cDNA (Superscript II GIBCO/BRL, Life Technologies) probe corresponding to RNA from the LTR6 cells incubated at 37°C and alternatively 4 hr at 32°C. The PCR product of a clone containing cyclin G was also spotted on each membrane and used as positive control. Slot blots were exposed overnight at −80°C.

**Quantitative Image Analysis.** Analyses were performed using the 1200 $\beta$ imager (Biospace Instruments, Paris) on both Northern blots (for TSIP1 and TSIP2) and slot blots for all the control cDNAs and TSAP1–8. For quantitative analysis represented by the graphics in Fig. 1, we have subtracted a constant number for each peak. This constant was calculated by measuring the mean value of the background noise in the slots that did not contain any cDNA. The $\beta$ imager results have been obtained by counting the slot blots overnight and have been confirmed by autoradiography with variable exposure times. These autoradiograms showed the same relative qualitative variation between activity at 32°C and at 37°C as the quantitative one measured with the $\beta$ imager. The $\beta$ imager was conceived following the principal of particle detectors with a PPAC (parallel plate avalanche chamber).

***In Situ* Hybridization (26, 27).** Cells were washed three times in phosphate-buffered saline (PBS), cytospinned, and fixed in 4% paraformaldehyde in PBS for 10 min and stored in 70% ethanol. Digoxigenin 11-uridine 5′-triphosphate (DIG) or biotin-11-UTP-labeled RNA transcripts from TSAP3 were used in the analysis following the procedures described

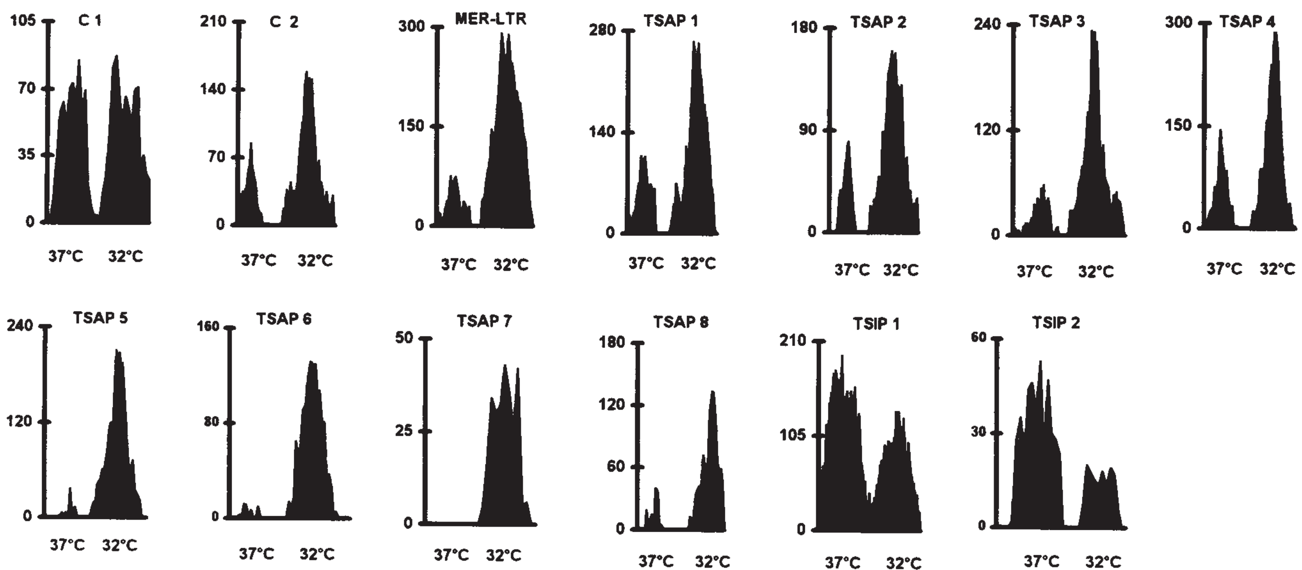


FIG. 1. Quantification of differential expression of mRNAs using the 1200 $\beta$ imager. Hybridization to mRNA derived from LTR6 cells at 37°C and from LTR6 cells after 4 hr at 32°C. Numbers in the ordinate, from 0 to 500, correspond to counts detected per 0.15 mm and are proportional to the hybridization signals. C1, equally expressed mRNA using a nondifferentially expressed clone from the comparative cDNA display; C2, positive control using cycline G shows induction of corresponding mRNA at 32°C; mouse endogenous retrovirus (MER)-LTR, induction of this sequence at 32°C; TSAP1–TSAP8, differential expression of the eight mRNAs activated in the first 4 hr after induction of apoptosis; TSIP1 and TSIP2, differential expression of the two mRNAs inhibited in the first 4 hr after induction of apoptosis.

(Boehringer Mannheim). For detection of hybridized digoxigenin-labeled probe, slides were incubated in SAD-10 (10-nm-gold-labeled-sheep anti-DIG antibodies, 1:1000 dilution; Biocell Laboratories) and silver enhanced. Analysis was performed by confocal scanning laser microscopy (MRC 600 Bio-Rad).

## RESULTS AND DISCUSSION

**Differential cDNA Display.** The PCR-based differential screening method by Liang and Pardee (1) provides an extremely powerful and efficient tool for detecting variations in gene expression. Nevertheless, any slight variation of the different experimental parameters induces unreproducible results. We therefore modified the original protocol. We found full reproducibility of the differential display in at least three separate experiments by introducing a hot start followed by a "Touch Down" (23). Differentially expressed bands were isolated and reamplified. In several cases, the reamplified product of a selected band contained contaminating bands originating from abundant mRNAs from both above and underneath migrating cDNAs. Such a probe used directly on Northern blots induces errors. We found that it was necessary to subclone the second PCR product and to perform the Northern blot analysis using as probe single recombinant clones. Systematic sequencing of at least 10 recombinant subclones for each selected band proved to be of great help in selection of clones of interest (data not shown).

**Differential Expression and Sequence Analysis.** The p53 gene is, at the present state of our knowledge, the tumor suppressor which is mutated in a high percentage of cancers of diverse origins (2, 3). The use of the Val-135 p53 temperature-sensitive mutant was shown in several studies to provide key information relative to wild-type function by inducing either growth arrest in the $G_1$ phase of the cell cycle or initiating programmed cell death (6, 7). The molecular pathways upstream and downstream of p53 leading to tumor suppression are still unclear. Several genes downstream of that for p53 have to date been identified including *gadd45* (9), *mdm2* (10, 11), *mck* (12), mouse endogenous retrovirus long terminal repeat (LTR) (15), p21-WAF1 (13, 14), and cyclin G (16).

In the present study, RNA was extracted 4 hr after activation of p53 at 32°C. Ten differentially expressed cDNA fragments were isolated, subcloned, sequenced, and studied by Northern blot and slot blot analysis. Hybridization signals were quantified with the 1200 $\beta$ imager using cDNA probes corresponding to the LTR6 mRNAs extracted after 4 hr of incubation at 37°C and at 32°C. Fig. 1 shows quantification of the hybridization signals corresponding to differential expression of eight TSAP and two TSIP clones. All eight TSAP clones show significant activation at 32°C; both TSIP clones show inhibition of expression at 32°C. Besides these 10 cDNAs, four other clones obtained in this differential cDNA analysis, correspond to the mouse endogenous retrovirus LTR family of genes (15).

The search for homologies revealed that three of the activated sequences, TSAP1–TSAP3, correspond to known genes (Table 1). The other cDNAs TSAP4–8 do not show any significant homology with known genes. The two cDNAs TSIP1 and 2, which are inhibited in their expression during apoptosis, show no homology with known genes. These genes, inhibited in their expression by wild-type p53, might eventually code for oncogenic sequences which would be downregulated in the process of tumor suppression or, alternatively, for structural and cytoskeletal proteins, for which downregulation of expression is concomitant with cell death by apoptosis.

TSAP1 is highly homologous to rat phospholipase C $\beta$4 (28, 29). The TSAP1 sequence presents 100% identity with phospholipase C between nucleotides 3967 and 3985, 82% between nucleotides 3986 and 4116, and 85% between nucleotides 4070 and 4220. Phospholipase C is known to be implicated in the signaling pathway of tyrosine kinase receptors (30) and to

Table 1. Characteristics of differentially expressed cDNA clones

| Differentially expressed clone | Primers 3′ and 5′* | mRNA, kb | Homology |
|---|---|---|---|
| TSAP1 | T11GC-16 | 2.0 and 4.5 | PLC† |
| TSAP2 | T11GC-5 | 5.9 | MEN1‡ |
| TSAP3 | T11CG-4 | 1.9 | siah1B§ |
| TSAP4 | T11GC-6 | 5.0 | No |
| TSAP5 | T11CG-5 | 1.2 | No |
| TSAP6 | T11AG-1 | 2.8 | No |
| TSAP7 | T11GC-16 | >8.0 | No |
| TSAP8 | T11GC-6 | >10.0 | No |
| TSIP1 | T11CG-8 | 3.0 | No |
| TSIP2 | T11AA-5 | 3.1 | No |

*Numbers and sequences of 5′ primers correspond to those reported by Bauer *et al.* (24).
†Rat phospholipase C $\beta$4 mRNA (RATPHOSCB).
‡Human mRNAs (HUMMEN1C, HUMZFM1C, HUMZFM1A, HUMMEN1A).
§siah1B mRNA (MMSIAH1B).

catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (31). Although phospholipase C has not been associated with tumor suppression, our finding might suggest that it is a downstream target in mediating the apoptotic effect of p53.

TSAP2 is highly homologous (92% identity between nucleotides 259 and 299, 100% identity between nucleotides 418 and 458, and 92% between nucleotides 645 and 685) to a zinc finger protein (ZFM1) that was localized in the multiple endocrine neoplasia (MEN1) locus (17). MEN1 is an autosomal dominant disorder associated with development of tumors affecting the anterior lobe of the pituitary, parathyroid gland, and pancreatic islet cells (18). Of great interest is the finding that both ZFM1 and an isoenzyme of phospholipase C were colocalized in the same chromosomal region 11q13 containing the susceptibility gene(s) to MEN1 (19). In mice, the homologous region is localized on chromosome 19B (32). The fact that we found TSAP1 and TSAP2 activated in response to p53

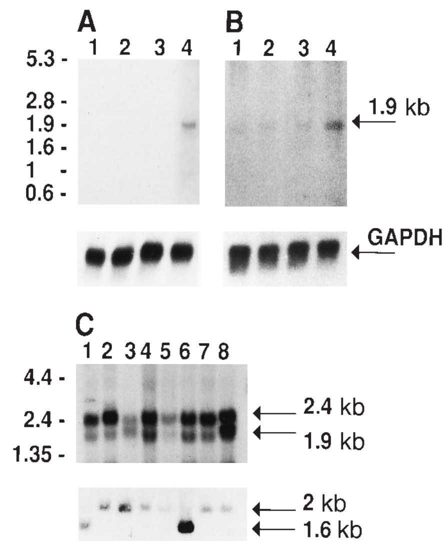


FIG. 2. Northern blot analysis. (*A*) Hybridization with TSAP3 probe. (*B*) Hybridization with mouse *siah1b* probe. Lanes 1 and 2, poly(A)$^+$ mRNA from M1 myeloid leukemia cells (clone S6) cultured at 37°C and 32°C, respectively; lanes 3 and 4, poly(A)$^+$ mRNA from LTR6 cells cultured at 37°C and 32°C, respectively. Arrow indicates differential expression of the 1.9-kb transcript TSAP3 mouse *siah1b*. (*A* and *B Lower*) GAPDH. (*C*) Tissue distribution using TSAP3 as a probe. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. Arrows indicate 1.9- and 2.4-kb transcripts. (*Lower*) $\beta$-Actin.

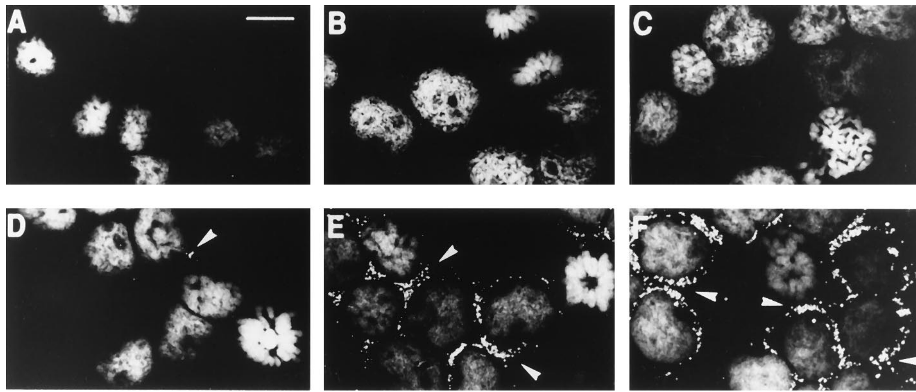


FIG. 3. *In situ* hybridization analysis with TSAP3 probe. (*A*) M1 cells incubated for 4 hr at 32°C and hybridized to TSAP3 antisense probe. (*B*) LTR6 cells incubated for 4 hr at 32°C and hybridized to TSAP3 sense probe. (*C*) LTR6 cells cultured at 37°C and hybridized to a TSAP3 antisense probe. (*D–F*) LTR6 cells cultured at 32°C for, respectively, 1, 2, and 4 hr and hybridized to the TSAP3 antisense probe. Arrowheads indicate accumulation of TSAP3 mRNA in cytoplasm. (Bar = 10 $\mu$M.)

might suggest that these genes belong to a more global pathway of tumor suppression and that p53 might cooperate with MEN1.

**TSAP3 Is Identical to *Siah1b*.** This gene is the vertebrate homologue (21) of *Drosophila* seven in absentia (*sina*) (20). Our clone presents 94% identity with the murine homologue (nucleotides 1496–1634). By Northern blot analysis, using as a probe TSAP3, we detect differential expression of the 1.9-kb messenger of this gene (Fig. 2*A*). This was confirmed by using a second probe corresponding to the same region of the reported *siah1b* sequence (21) (Fig. 2*B*). Fig. 2*C* shows the tissue distribution of this gene by using a TSAP3 probe that detects both the 1.9- and the 2.4-kb mRNAs corresponding to previously reported results where a *siah* probe was used (21). *In situ* hybridization (Fig. 3) shows that TSAP3 mRNA poorly expressed after 1 hr (Fig. 3*D*) is clearly detected 2 hr after induction of apoptosis (Fig. 3*E*). Its expression strikingly increases between 2 and 4 hr (Fig. 3 *E* and *F*). In the cells that had entered mitosis, no signal was detected.

Carthew and Rubin (20) have shown that seven in absentia is required for specification of R7 cell fate in *Drosophila* eye. Furthermore, mutants of this gene in *Drosophila* show a disrupted adult sensory bristle formation, have a lethargic adult behavior, and show subviability and infertility, suggesting a more general role in development. Although *sina* is downstream of the tyrosine kinase receptors and Ras-1 signaling, it remains unknown precisely how this gene is activated (33, 34). The murine homologue is subdivided in two groups, *siah1* and *siah2.* These proteins show an unusually high degree of conservation with *Drosophila* seven in absentia (21). Our data show that TSAP3/*siah1b* is activated in programmed cell death in M1 cells induced by the p53 tumor suppressor gene. Since this gene encodes a nuclear zinc finger protein, it could be a regulatory transcription factor that is downstream of the p53 signal. These data also provide a direct link between a developmentally conserved *Drosophila* gene and a major pathway of tumor suppression.

While the link between oncogenes activated in human tumors and developmental genes in *Drosophila* is firmly established, this is not yet entirely the case between the confirmed human tumor suppressor genes. At least this is not the case for tumor suppressors such as RB, p53, WT-1, NF-1, VHL, APC, and DCC, aside from NF-2, which belongs to the 4.1 family of membrane-cytoskeletal linker molecules and is part of the expanded locus in *Drosophila* (35). However, this is a fast-growing area and with identification of 50 tumor suppressors in *Drosophila* (36) there is no doubt that those evolutionary conserved genes are extremely important in understanding tumor suppression.

In conclusion, we have cloned 10 cDNAs, which provide us with previously unrecognized molecules involved in the signaling pathway of p53. Finally, the advantage of coupling a functional model system such as M1-LTR6 to a powerful global technology for screening of gene expression like the differential cDNA display lies in the fact that it provides a direct functional link between the biological model system and the cDNA sequence cloned.

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