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Research Article

Identification of functional genes during Fas-mediated apoptosis using a randomly fragmented cDNA library

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Abstract. We describe a general strategy for the identification of functional genes that, when downregulated, result in a selectable phenotype. This strategy is based on expression selection of cDNA fragments that counteract their cognate genes. A cDNA library containing random fragments expressed in human HepG2, A375 and CLS-354 cells was used to identify functional genes whose inhibition conferred resistance to Fas-induced apoptosis. Thirty-five clones were isolated, 28 of which were derived from unknown genes, that tagged 19 individual genes and 7 of which referred to known genes that tagged the apoptosis-related protein (APR)-1, -2 and indoleamine-pyrrole 2,3,-dioxygenase (IDO). The ability of APR-1-, -2- and IDO-derived antisense RNAs to induce resistance to Fas in HepG2, A375 and CLS-354 cells suggested that APR-1, -2 and IDO genes are involved in the machinery of Fas-mediated apoptosis. Our gene discovery strategy provides a generally applicable procedure to identify functional genes that interfere with apoptosis, and may therefore be clinically relevant for tumor therapy.

Key words. Apoptosis; functional gene; cDNA library; APR-1; APR-2; IDO.

Cell killing by cytotoxic agents is a complex process regulated by many different genes. The outcome of treatment with a cytotoxic agent is determined by the balance between the activity of the antiapoptotic and proapoptotic genes, whose function is essential to cytotoxic agent-mediated cell killing [1, 2]. Expression selection has often been used to isolate genes whose mutation or overexpression results in a dominant selectable phenotype. Many phenotypes, however, are derived from downregulation rather than overexpression as well as dominant mutations of specific genes. In addition, genes expressed at low levels are underrepresented in both conventional and antisense cDNA libraries, and their isolation is further complicated by the limited efficacy of DNA transfection used in standard expression selection protocols.

Up to now, genomic information has been used mainly to develop expression array technologies. The general weakness of this approach is that expression profiling gives no indication of gene function and is, therefore, not suited for the functional annotation of the human genome. As such, expression arrays have been useful in defining prognostic profiles for a given form of cancer [3]. However, they have rarely permitted insight into potential therapeutic strategies.

Since metastasis of different tumor cells is often resistant to standard treatment, it requires novel therapeutic strategies targeting relevant intracellular processes. In this re-

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gard, the identification of functional human genes that potentiate the cytostatic or cytotoxic response to anticancer agents is of crucial importance. The yeast two-hybrid system has been widely used for the identification of functional genes. The yeast two-hybrid system is a powerful method for the detection of direct interactions between proteins in vivo and for the identification of genes whose products interact with proteins derived from a cDNA library. The method is limited by the fact that the interaction does not always reflect a biological role and that false-positive results are often obtained. Much progress has been made in the identification of genes that can be identified by positive expression selection [4]. Analysis of recessive genes (e.g. proapoptotic/tumor suppressor genes) is technically more challenging and is made amenable by the development of a functional genetic approach [5]. This functional genetic approach is based on the expression of cDNA fragments that act as antisense RNAs resulting in the inhibition of gene expression. Because these antisense RNA segments behave as dominant selectable markers for the phenotype associated with the repression of the respective gene [6], they are suited for the identification of functional genes (e.g. proapoptotic/tumor suppressor genes) that may have relevance for tumor therapy.

In the present work, we used a normalized cDNA library expressing random RNA fragments to isolate clones conferring resistance to Fas-induced apoptosis. Thirty-five cDNA fragments encoding antisense RNA segments were isolated using this gene discovery procedure and further characterized.

Material and methods

Construction of randomly fragmented cDNA libraries

The cDNA libraries were generated from a mixture of RNAs (~5 µg) harvested at regular time intervals up to 48 h from human hepatocarcinoma HepG2 cells before and after treatment with the agonistic anti-Fas antibody CH11 (500 ng/ml; Immunotech) so that both constitutive and CH11-induced mRNA transcripts were included. Double stranded (ds) cDNA was generated using the cDNA synthesis kit according to the manufacturer's protocol (Clontech). The ds cDNA products were pooled and subjected to partial DNase I (Roche) digestion as described elsewhere [7]. The reaction was stopped by the addition of EDTA to a final concentration of 0.25 mM at different time points to obtain cDNA fragments of various average length (between 200–700 bp). Randomly fragmented cDNAs were blunted with T4 DNA polymerase and Klenow fragment (Roche) and directly ligated with an excess of pCEP4 vector (Invitrogen) that had been digested with PvuII to generate a blunt-end cloning site. The amount of DNA used for ligation was adjusted so that more than 1×10^5 colonies could be obtained. Plasmid DNA was prepared directly from pooled bacterial colonies to avoid an amplification step. PCR analysis of the resulting library confirmed that the insert size remained unchanged.

Cell culture and transfection procedures

Human hepatocellular carcinoma, cell line HepG2 (ATCC), human melanoma cell line A375 and human oral squamous carcinoma cell line CLS-354 that constitutively express the human Fas gene were grown in DMEM medium supplemented with 10% FCS (Sigma), 2 mM L-glutamine and 100 units/ml penicillin/streptomycin (GIBCO). Transfection was performed by the nucleofector method (Amaxa). Briefly, HepG2, A375 or CLS-354 cells were plated 1 day before transfection so that cells were about 70% confluent on the day of transfection. Following one wash with cold PBS, cells were detached from the dish and then washed twice with PBS. The density was adjusted to 1×10^6 cells that were used for transfection with 5 µg of the plasmid carrying the library DNA. After transfection, the cells were plated in culture dishes and allowed to grow for 48 h before starting selection.

Selection of functional genes

In the present study, two selection strategies were used to isolate anti-Fas-resistant cells. In the first protocol, 1×10^6 HepG2 cells were transfected with the library DNA, and 48 h after transfection exposed to 500 ng/ml agonistic anti-Fas antibody and hygromycin 300 µg/ml (Clontech) for 4 weeks. Individual surviving colonies were picked and expanded. In the second protocol, the cells were first selected with hygromycin (300 µg/ml), and surviving colonies were expanded for 4–6 weeks. Hygromycin-resistant cells were then plated at 1×10^5 per 100 mm plate in medium containing 500 ng/ml CH11. After 2 weeks of selection with CH11, cells from surviving colonies were collected and episomes were extracted as described previously [8].

Identification of genes conferring resistance to Fas-mediated apoptosis

A total of 35 clones (120 antisense cDNA fragments), obtained after two independent selection experiments, were tested in baches (mixed clones with multi-cycle selection procedure) or individually (individual clones with one-cycle selection procedure) for the ability to confer resistance to Fas-mediated apoptosis after introduction into HepG2, A375 and CLS-354. In batch testing, the cells (1×10^5 per 100-mm plate, each) were transfected with pooled clones purified from the surviving HepG2 cell population. After 2 weeks of selection with CH11, surviving colonies were picked and episomes were purified. Then, isolated episomes were introduced into *Escherichia coli* for limited amplification. After purification of am-

plified episomes they were re-introduced into target cells and this procedure was repeated five times, prior to plating on a LB agar plate. Similarly, individual clones obtained from batch testing were examined individually in HepG2, A375 and CLS-354 cells for the ability to confer resistance to Fas-mediated apoptosis. The clones were sequenced and analyzed for homology using the BLAST search program [9].

Assessment of cell survival

The percentage of viable cells was determined using the colorimetric MTT assay (Roche) as described elsewhere [10, 11].

Detection of apoptosis

The percentage of apoptotic cells was determined by TUNEL analysis (Roche) as described elsewhere [12]. Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized by treatment with 0.1% Triton X-100, washed with PBS and incubated in 1× terminal deoxy-nucleotidyltransferase (TdT) buffer that contained 300 U/ml TdT and 40 µM biotin-dUTP for 60 min at 37 °C according to the manufacturer's protocol. Cells were then washed with PBS. TUNEL-positive cells were detected by incubation with fluorescein isothiocyanate-conjugated streptavidin for 30 min at 37 °C.

RT-PCR

Total RNA was isolated from HepG2, A375, and CLS-354 cells using the RNeasy kit (Qiagen) according to the manufacturer's protocol. RT-PCR was performed using a RNA PCR kit (Qiagen) with Fas upstream (5′-ACT GGC TCA AAA CTA CCT ACT TC-3′) and downstream (5′-CTT AGA AAC TTG GGG GTA TGA CA-3′) primers or GAPDH primers (Promega) as control.

Immunoblot

Immunoblot analysis was performed according to standard procedures. The following antibodies were used at the indicated dilution: anti-IDO antibody 1:2000 (Biogenes); anti-PARP antibody, 1:2000 (#9542, Cell Signaling); anti-actin, 1:5,000 (SC-1615, Santa Cruz Biotechnology).

Results

Functional analysis of randomly fragmented cDNA libraries

We first investigated the expression of the Fas receptor in HepG2, A375 and CLS-354 cells. Using conventional RT-PCR, we could demonstrate the expression of Fas receptors on all cells (fig. 1A). In addition, we determined the sensitivity of cells bearing Fas receptors to the agonistic anti-Fas antibody CH11. Cells (HepG2, A375 and CLS-354) were challenged with 500 ng/ml CH11 and cell survival was estimated at various time intervals up to 96 h using the MTT assay. As shown in figure 1B, cell death occurred in HepG2 after 36 h, in A375 after 48 h, and in CLS-354 after 72 h. To confirm that the Fas-induced death of HepG2, A375 or CLS-354 cells was mediated through apoptosis, cells were treated with CH11 for the indicated periods and then lysed. Cellular proteins were subjected to Western blot analysis of PARP cleavage (fig. 1C). The cleavage of PARP in response to the treatment with CH11 demonstrated that Fas-induced death of HepG2, A375 and CLS-354 cells was regulated by an apoptotic mechanism.

Next, we prepared a library of ~1×10^6 clones containing 200- to 700-bp fragments (data not shown) from a mixture of RNAs harvested at regular time intervals up to 48 h from HepG2 cells before and after treatment with the agonistic anti-CD95 antibody CH11 (500 ng/ml; Immunotech), so that both constitutive and CH11-induced mRNA transcripts were included. The library was constructed in pCEP4 (Invitrogen), an EBV-based episomal vector (fig. 2A). The cDNA fragments were generated by incomplete DNase I digestion and ligated into the PvuII site of the vector. Because the fragmentation and subcloning direction were both random, we assumed that half of the fragments were inserted in an antisense orientation. The choice of an episomal shuttle vector provided two major advantages: high efficiency of stable transfection without requiring chromosomal integration; reduced appearance of false-positive clones that could result from the HepG2 cells. The randomized cDNA library was introduced into HepG2 cells and subjected to two different selection strategies to isolate anti-Fas-resistant cells (fig. 2B). The isolated episomes containing cDNA fragments were subsequently tested in batches or individually as shown (fig. 2B). Cell colonies that survived the prolonged selection were pooled, episomes were isolated and used to transform bacteria. More than 120 isolated clones were amplified and their cDNA inserts were sequenced. Sixteen fragments appeared only once, tagging at least ten independent genes, and the rest appeared multiple times corresponding to 19 different clones that tag three known genes (28 fragments) including two apoptosis-related proteins (APR-1 and APR-2) and indoleamine 2,3-dioxygenase (IDO) as well as 9 unknown genes (76 fragments). When we individually tested these identified clones for their ability to confer resistance to Fas-induced apoptosis in HepG2, A375 or CLS-354 cells, we could demonstrate that 18 of the isolated fragments were able to confer resistance to apoptosis in all tested cell types (table 1). These fragments tagged at least 11 independent genes including APR-1, -2 and IDO, the inactivation of which generates resistance to Fas-induced apoptosis, illustrating the complexity of this phenotype. Seven of the isolated fragments were unable to confer resistance in A375 (table 1). These fragments tagged three genes whose inactivation in HepG2 and CLS-354 cells led to re-

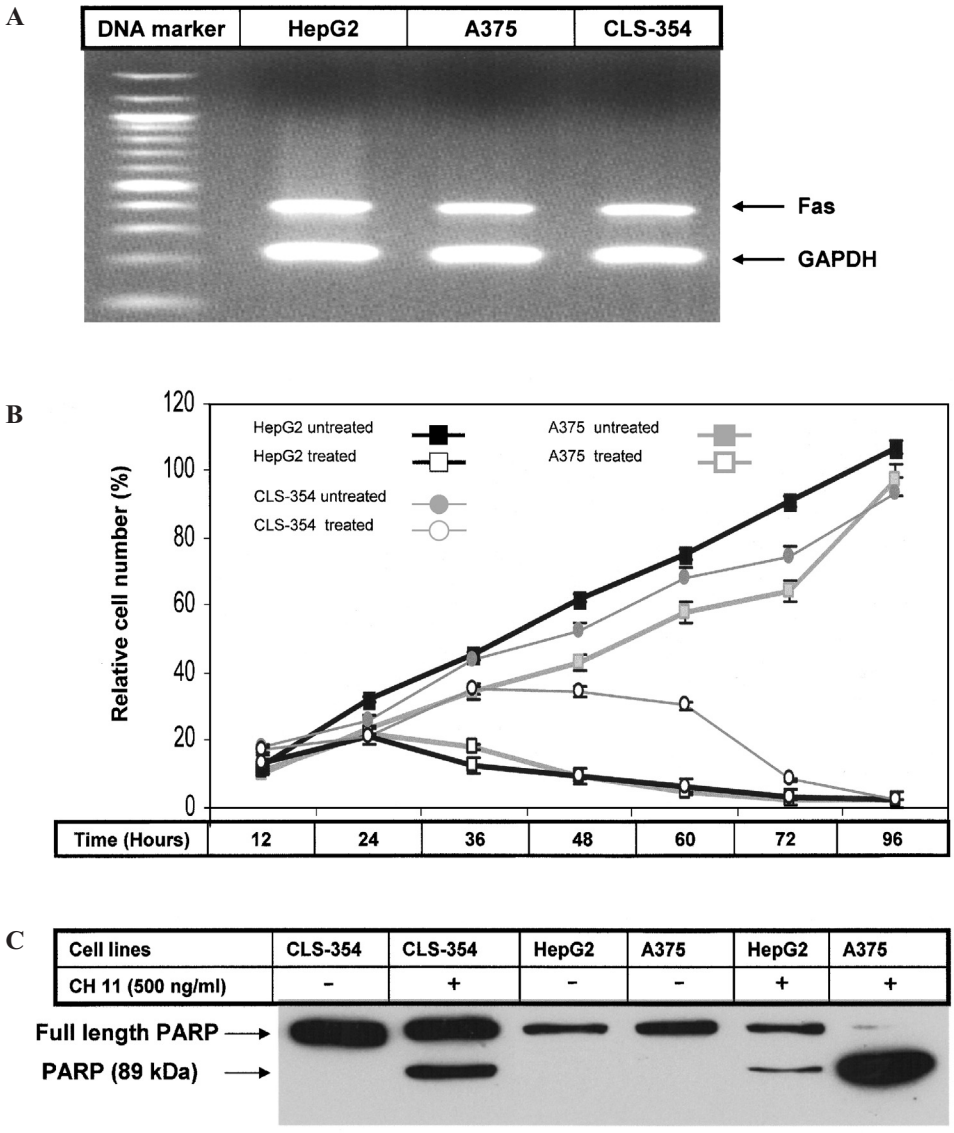


Figure 1. Sensitivity of the tumor cell lines HepG2, A375 and CLS-354 to anti-Fas antibody-induced cell death. Cells were treated with CH11 for the indicated time points. (*A*) Constitutive expression of Fas receptor in HepG2, A375 and CLS-354 cells using semi-quantitative RT-PCR. (*B*) Percentage of cell survival after treatment with the agonistic anti-Fas (CH11) at different time periods up to 96 h. Exponentially dividing cells were treated with 500 ng/ml anti-Fas antibody. Cell viability was determined using the MTT assay. Depicted are the means ± SD from duplicate experiments. (*C*) PARP cleavage after the treatment with CH11 for 48 h using Western blot analysis.

sistance to Fas-induced apoptosis. In addition, 9 of the isolated fragments were unable to confer resistance in CLS-354 (table 1). These fragments tagged 6 genes whose inactivation generated resistance to Fas-induced apoptosis in HepG2 and A375 cells, whereas one of the isolated cDNA fragments (clone XVIII) was unable to confer resistance to Fas-induced apoptosis in A375 and CLS-354 cells (table 1).

Characterization of identified cDNA fragments conferring Fas resistance

The sequences of the cloned cDNA fragments were analyzed for homology to known nucleic acid and protein sequences (table 1). A high homology was found to the apoptosis-associated genes apoptosis-related protein-1, -2 (APR-1, -2) and indoleamine 2,3-dioxygenase (IDO). Although sequence analysis revealed a high degree of similarity between the sequences of the identified fragments and reported sequences in GenBank (table 1), comparison of the additional sequences did not correspond to known genes.

To investigate whether the apoptotic functions were correlated with a given phenotype in our gene discovery system, we examined the relative numbers of apoptotic cells transfected with functionally identified cDNA fragments during apoptosis induced by CH11 using the TUNEL method

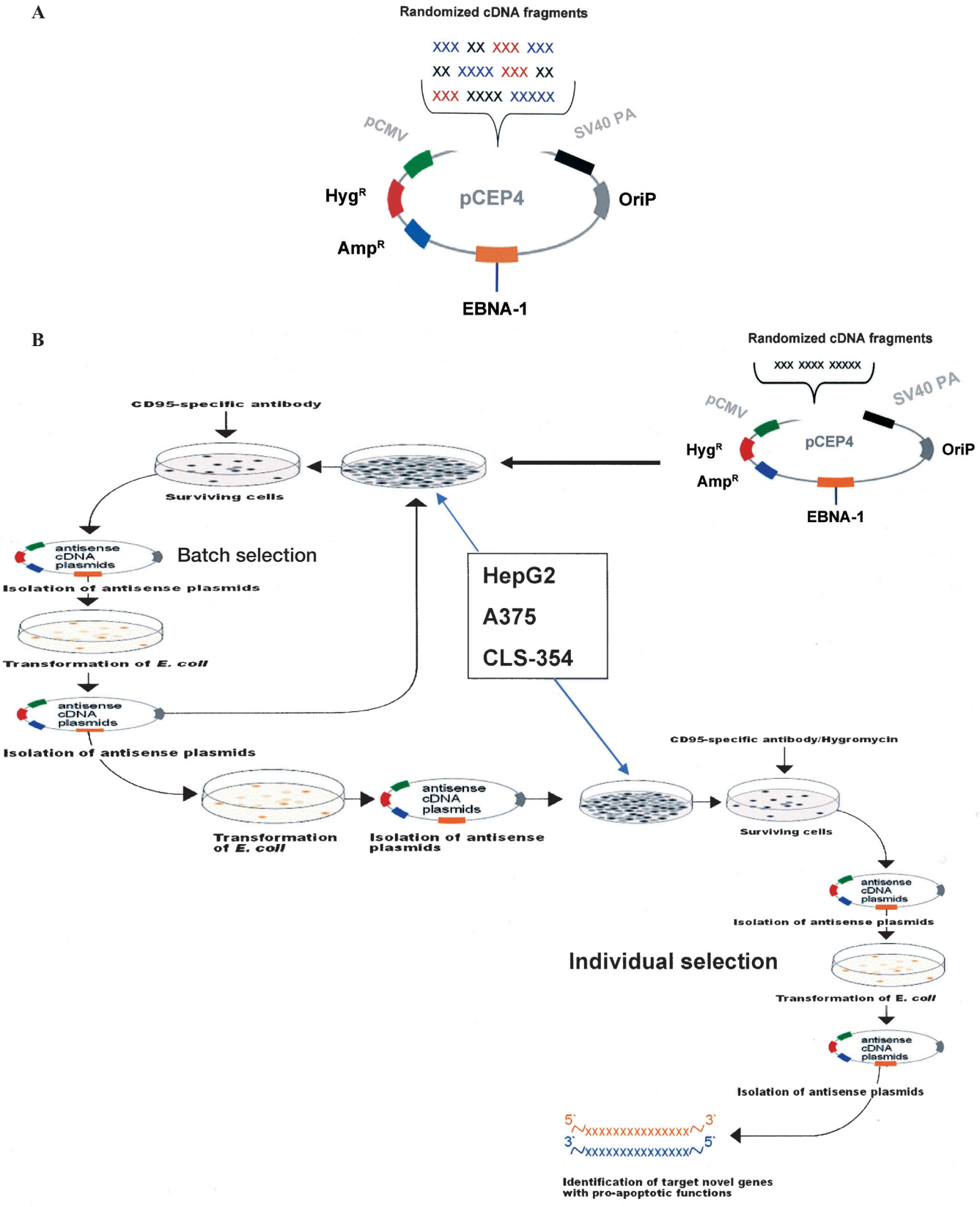


Figure 2. Rationale for identification of genes that act in Fas-mediated apoptosis using a randomly fragmented cDNA library. (*A*) Construction of plasmids that contained a randomly fragmented cDNA library. Hyg^R, hygromycin; Amp^R, ampicillin; EBNA-1, Epstein-Barr virus nuclear antigen type, coding sequence; OriP, episomal origin of replication; SV40 PA, splice and polyadenylation signal derived from SV40. (*B*) Schematic diagram of the gene discovery system (in batch and individual selection).

Table 1. Properties of identified cDNA fragments.

cDNA fragment	Size (bp)	Frequency[a]	Resistance[b] HepG2	A375	CLS-354	Effect	mRNA[c] (kb)	Gene name	Acc. No.
I	310	32	+++	+++	+++	antisense	0.444	unknown	AI290216
II	160	2	+++	+++	+	antisense	1.475	IDO	NM_002164
III	410	9	+++	+++	+	antisense	1.475	IDO	NM_002164
IV	720	4	+++	++	0	antisense	0.756	unknown	BE614298
V	470	3	++	++	0	antisense	0.595	unknown	BF475678
VI	610	2	++	++	++	antisense	–	unknown	AP001172
VII	180	2	+++	+++	+++	antisense	0.616	APR-1	CA948027
VIII	290	4	++	++	++	antisense	0.616	APR-1	CA948027
IX	425	1	+	+	+	antisense	–	unknown	AP001172
X	435	1	++	++	0	antisense	0.756	unknown	BE614298
XI	448	1	++	0	+++	antisense	0.756	unknown	BE614298
XII	645	1	+	+	+	antisense	–	unknown	AL365364
XIII	455	1	+++	+++	+++	antisense	–	unknown	AL365364
XIV	285	4	++	+++	++	antisense	0.791	APR-2	AF143236
XV	210	2	++	+++	+	antisense	0.791	APR-2	AF143236
XVI	270	5	++	+++	+++	antisense	0.791	APR-2	AF143236
XVII	435	6	++	++	++	antisense	–	unknown	AC005076
XVIII	690	3	++	0	0	antisense	–	unknown	AC005076
XIX	250	1	++	++	++	antisense	0.393	unknown	Al185076
XX	200	1	+	++	++	antisense	0.393	unknown	Al185076
XXI	570	1	+	0	++	antisense	–	unknown	AC093028
XXII	440	5	++	++	+++	antisense	0.548	unknown	AU144472
XXIII	435	3	+	+	+	antisense	–	unknown	AY566253
XXIV	690	1	++	0	+++	antisense	–	unknown	AY566253
XXV	250	1	++	++	++	antisense	4.200	unknown	AK127097
XXVI	200	1	++	++	0	antisense	4.200	unknown	AK127097
XXVII	570	1	+	+	0	antisense	–	unknown	AL355796
XXVIII	440	7	++	0	++	antisense	–	unknown	AL355796
XXIX	440	6	++	0	++	antisense	0.466	unknown	AI354965
XXX	435	3	++	0	+	antisense	–	unknown	AL356059
XXXI	690	1	++	++	++	antisense	0.861	unknown	AL528981
XXXII	250	1	+++	+++	+++	antisense	0.759	unknown	AV710703
XXXIII	200	1	++	++	0	antisense	0.386	unknown	AA120832
XXXIV	570	1	++++	+++	0	antisense	–	unknown	AL122016
XXXV	440	2	++	++	0	antisense	1.175	unknown	BU161481

[a] Number of clones in which each antisense was found.
[b] Ability of each antisense to confer Fas resistance to HepG2, A375 or CLS-354 cells was scored as positive when the number of surviving clones was at least twice as much as that in the control (HepG2, A375 or CLS-354 cells transfected with insert-free vector).
[c] Size of mRNAs of the identified clones.

(fig. 3A–C). Cells stably transfected with cDNA fragments exhibited significantly reduced apoptosis, whereas control cells transfected by insert-free vector readily underwent programmed cell death (fig. 3A–C). These results suggested that the phenotypes of cells that expressed the antisense RNA segments, anti-APR-1, anti-APR-1, or anti-IDO correlated with those of positive clones obtained by our functional gene discovery procedure, demonstrating the usefulness and validity of the described procedure.

Phenotypic effects of anti-IDO RNA segments in tumor cells

We next analyzed the expression of IDO in the tumor cell lines HepG2, A375 and CLS-354 before and after treatment with the anti-Fas antibody. In Western blot analysis, we could demonstrate the expression of IDO in all cell lines (fig. 4A). Interestingly, treatment of the same cell lines with anti-Fas antibody increased the expression levels of IDO in both HepG2 and CLS-354, but not in A375. To confirm the ability of anti-IDO RNA segments to confer resistance to Fas-mediated apoptosis in the tumor cell lines (HepG2, A375 and CLS-354), the cell lines were transfected with either insert-free or anti-IDO-pCEP4. As illustrated in figure 3A–C, cell populations transfected with anti-IDO showed a pronounced increase in the proportion of cells surviving Fas-induced apoptosis. Interestingly, cells stably expressing anti-IDO RNA segments (clone II or clone III) resisted apoptosis mediated by CH11 and showed a significant decrease in the expression levels of IDO in HepG2 and A375 cells

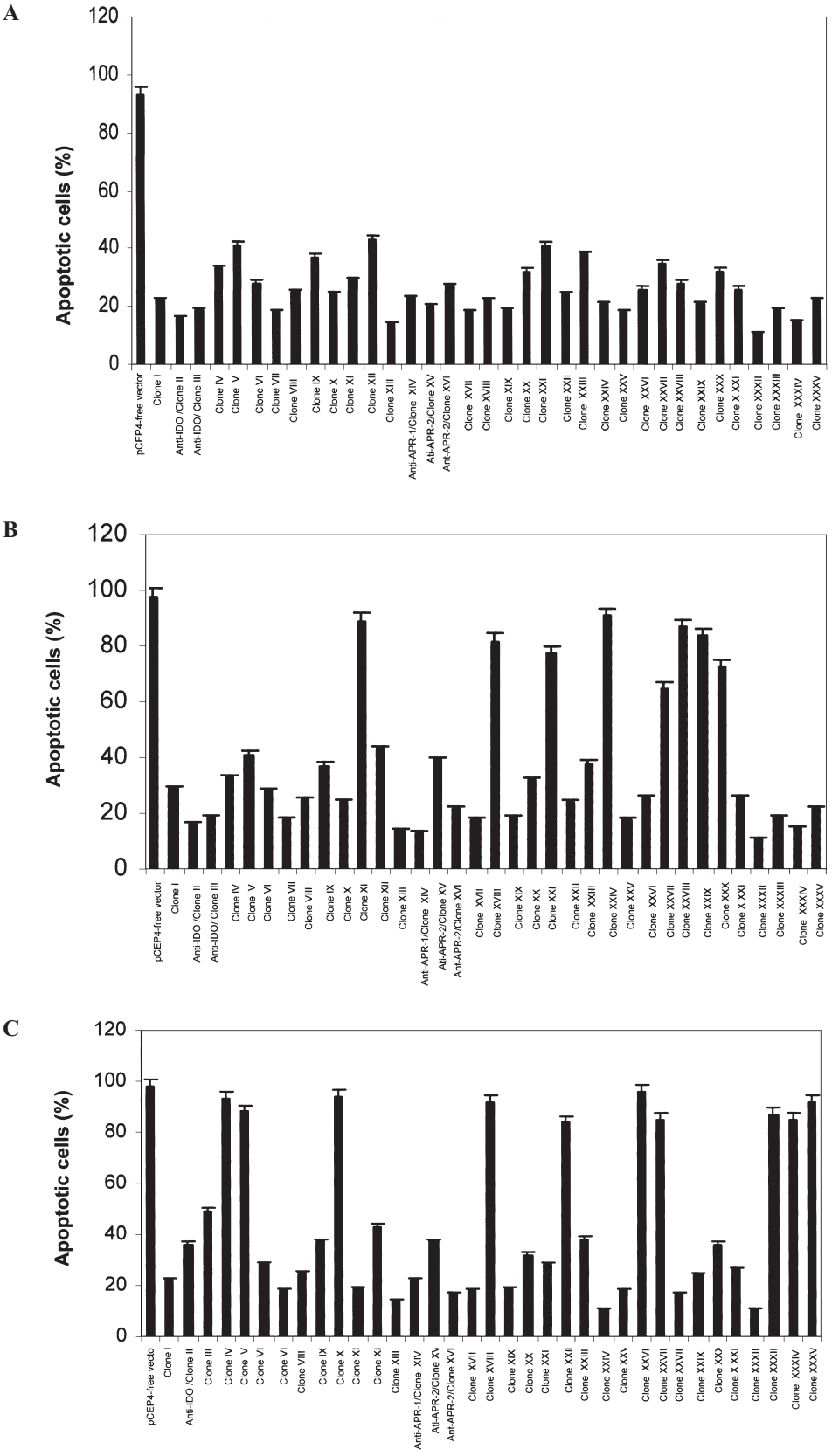


Figure 3. Apoptotic function of target genes. Extent of apoptosis 96 h after treatment with CH11 in populations of HepG2 (*A*), A375 (*B*) and CLS-354 (*C*) cells. Expression of insert-free pCEP4 or of pCEP4 carrying cDNA fragments directed against each gene (means ± SD of three experiments).

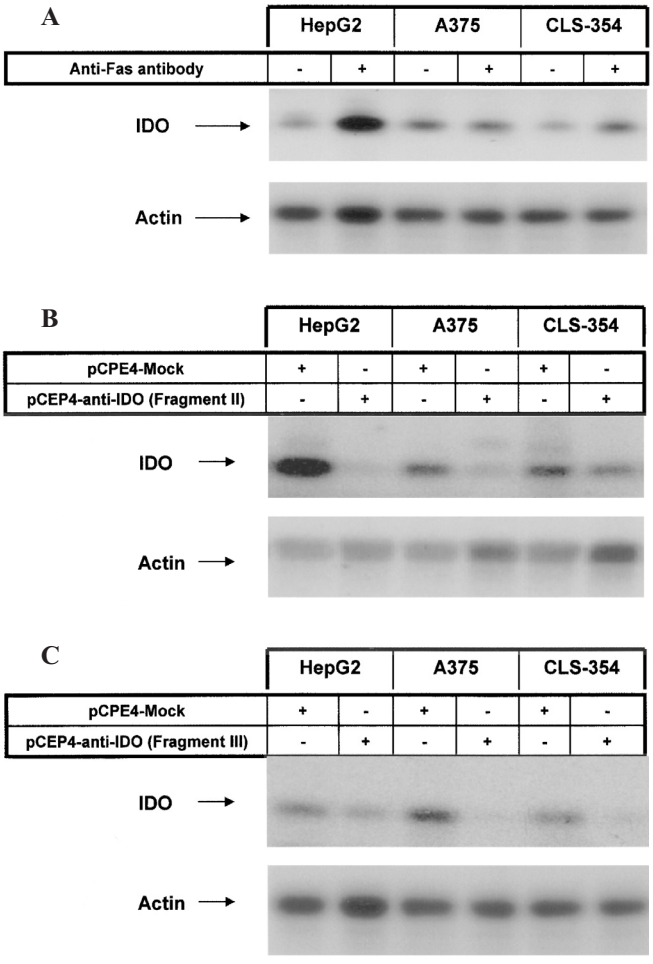


Figure 4. Levels of IDO protein in HepG2, A375 and CLS-354 cells stably expressing insert-free vector or anti-IDO clones using immunoblotting. (*A*) Equal amounts of whole-cell lysates (50 µg) were subjected to immunoblot analysis using are anti-IDO antibody. (*B*) Cells expressing either insert-free vector or clone II. (*C*) Cells expressing either insert-free vector or clone III. The same blots were reprobed with an anti-actin antibody to compare loading and transfer. These results are representative of three independent experiments.

(fig. 4B,C), whereas, the expression of clone III, conferred only moderate resistance to Fas in CLS-354 cells. Although the basal expression level of IDO does not appear to be influenced by the treatment of A375 cells with anti-Fas antibody, the suppression of IDO expression and the subsequent inhibition of anti-Fas-induced apoptosis in A375 cells upon expression of anti-IDO confirmed the importance of the IDO protein in the machinery of Fas-mediated pathways leading to apoptosis.

Induction of IDO by interferon-γ and lack of increased sensitivity to Fas-induced apoptosis

We next evaluated whether interferon (IFN)-γ increased IDO expression in HepG2, A375 and CLS-354 cells (fig. 5A). IFN-γ (1000 U/ml) showed a time-dependent induction of IDO expression in all three analyzed cell lines with maximal levels being detected at 72 h. We further examined the effect of IDO overexpression towards the sensitivity of Fas-induced apoptosis. The cell lines were pretreated with IFN-γ for 72 h and then incubated with the CH11 antibody (500 ng/ml). Forty-eight hours later, cell viability was measured using the MTT assay (number of viable cells; fig. 5B–D). Results obtained from MTT assays revealed that the induced (over-)expression of IDO had no significant effect on the sensitivty of Fas-induced apoptosis in HepG2 (fig. 5B), A375 (fig. 5C) or CLS-354 (fig. 5D) cells (also refer to fig. 1C).

Specificity of anti-IDO-mediated resistance to Fas-induced apoptosis

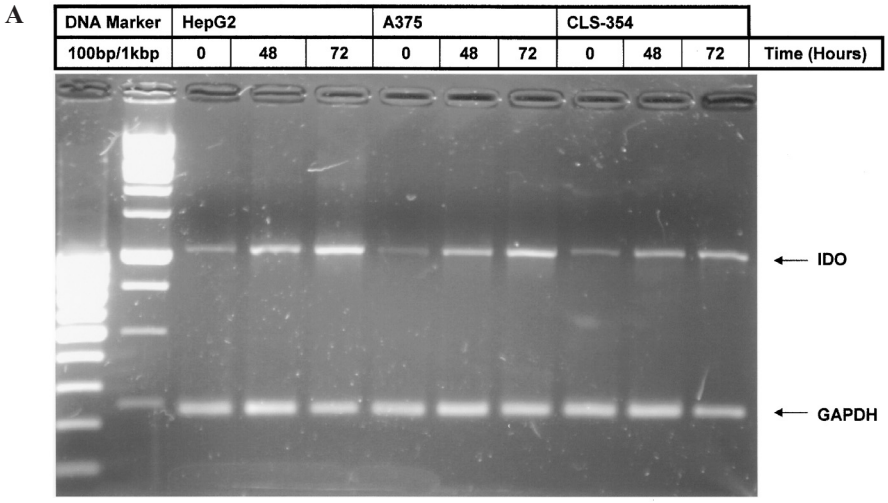
To examine whether the anti-IDO protective effect was specific for Fas-induced cell death, we treated HepG2, A375 and CLS-354 cells stably expressing anti-IDO antisense cDNAs (II and III) with tumor necrosis factor (TNF)-α (10 ng/ml) for 48 h as well as with cisplatin (100 µM) for 12 h. We measured cell viability following treatment of the cell lines for the indicated time points using the MTT assay (fig. 6A–C). Data obtained from the performed experiments showed that in addition to their protective effect associated with Fas-induced cell death, the anti-IDO II and III conferred resistance only to TNF-α-mediated cell death but not to cisplatin-mediated cell death in HepG2, A375 and CLS-354 cells (fig. 6A–C). These data provide evidence that the anti-IDO mediated protective effect may be restricted to death-receptor-mediated cell death, but not to chemotherapy-mediated cell death.

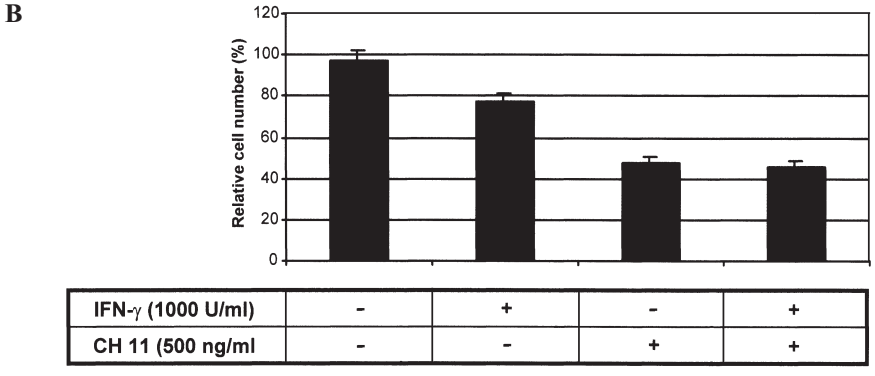
Pretreatment with a pharmacological inhibitor of IDO confers resistance to Fas-mediated cell death

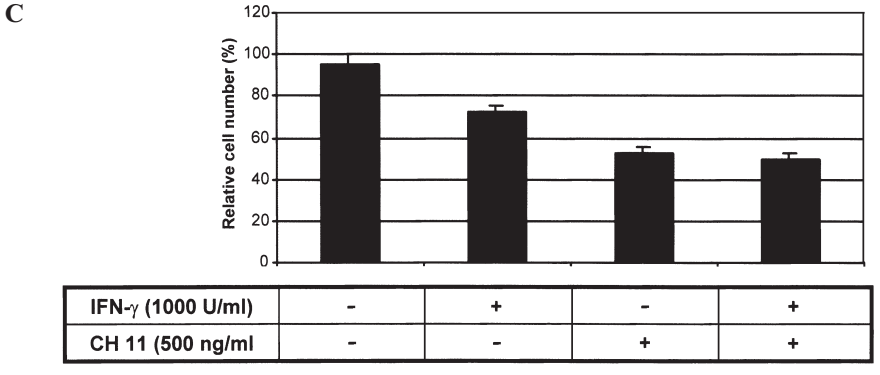
To examine whether the inhibition of IDO confers resistance to Fas-induced apoptosis, the cell lines HepG2, A375 and CLS-354 were pretreated with 1-MT, an inhibitor of IDO, for 72 h prior to exposure against CH11. Using 1-MT, resistance to Fas-mediated cell death was observed in all tested cell lines (fig. 7A–C).

Discussion

Selection of antisense RNA segments has the ability to directly identify genes that are functionally related to a given phenotype. Using this approach we identified several novel genes exerting inhibitory functions in Fas-mediated apoptosis. When we individually tested the identified clones for their ability to confer resistance to Fas-mediated apoptosis in HepG2, A375 or CLS-354 cells, we could demonstrate that about half of the isolated fragments were able to individually confer resis-







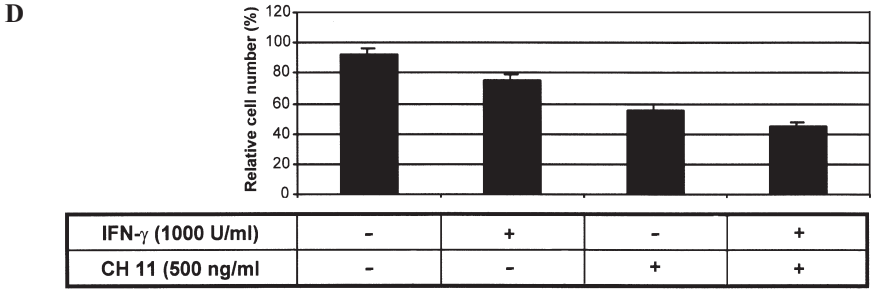


Figure 5. Time course of IFN-γ-induced expression of IDO in HepG2, A375 and CLS-354 cells. (*A*) Time-dependent induction of IDO expression in all three cell lines. (*B–D*) MTT assays revealed that the IFN-γ-induced (over-)expression of IDO had no significant effect on the sensitivty to Fas-induced apoptosis in HepG2 (*B*), A375 (*C*) or CLS-354 (*D*) cells.

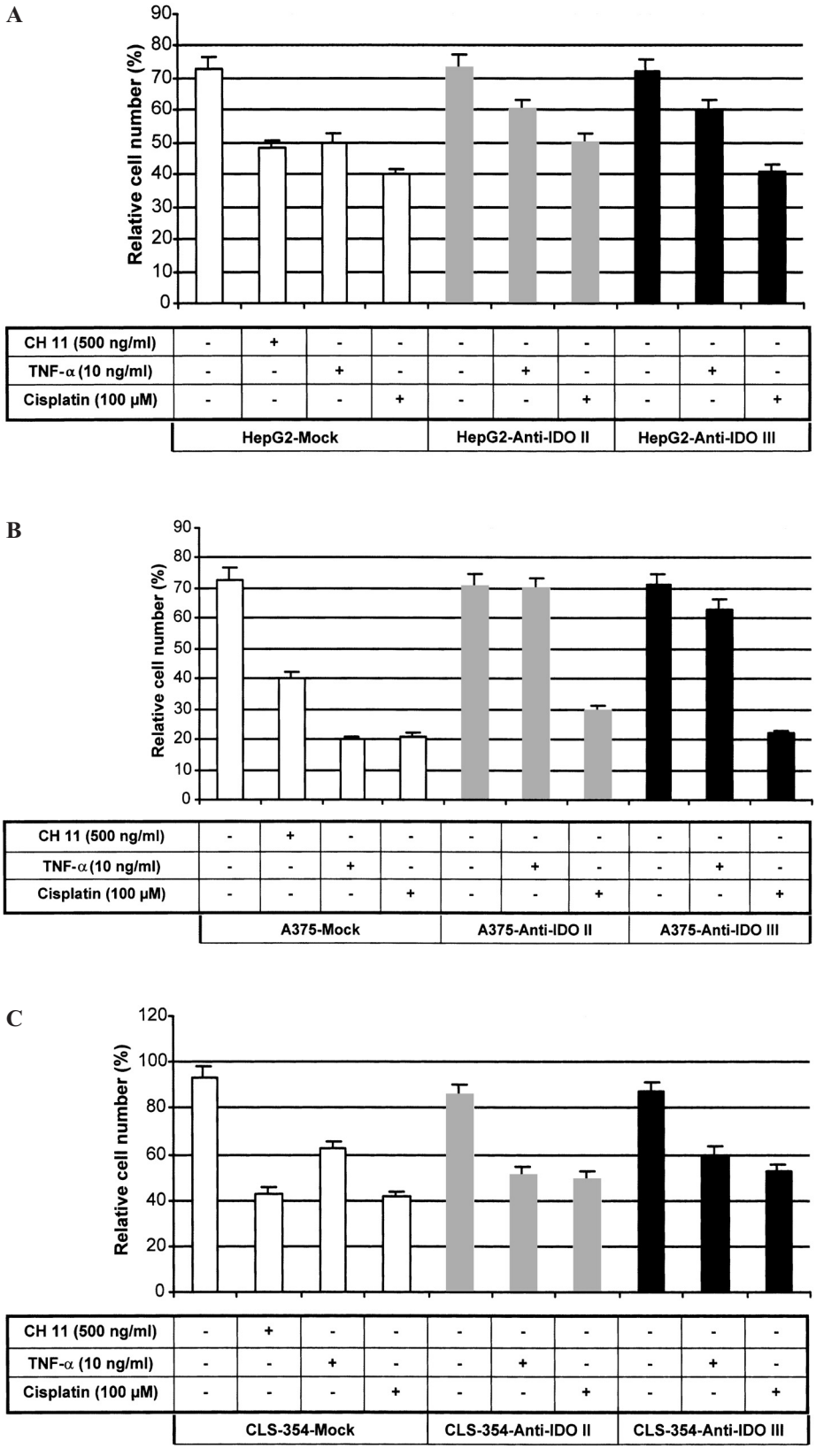


Figure 6. Specificity of anti-IDO-mediated resistance to Fas-induced apoptosis. To examine whether the anti-IDO protective effect was specific for Fas-induced cell death, we treated HepG2, A375 and CLS-354 cells stably expressing anti-IDO antisense cDNAs (II and III) with TNF as well as with cisplatin. The anti-IDO II and III fragments were found to confer resistance to TNF-α -mediated cell death but not to cisplatin-mediated cell death in HepG2 (*A*), A375 (*B*) and CLS-354 cells (*C*).

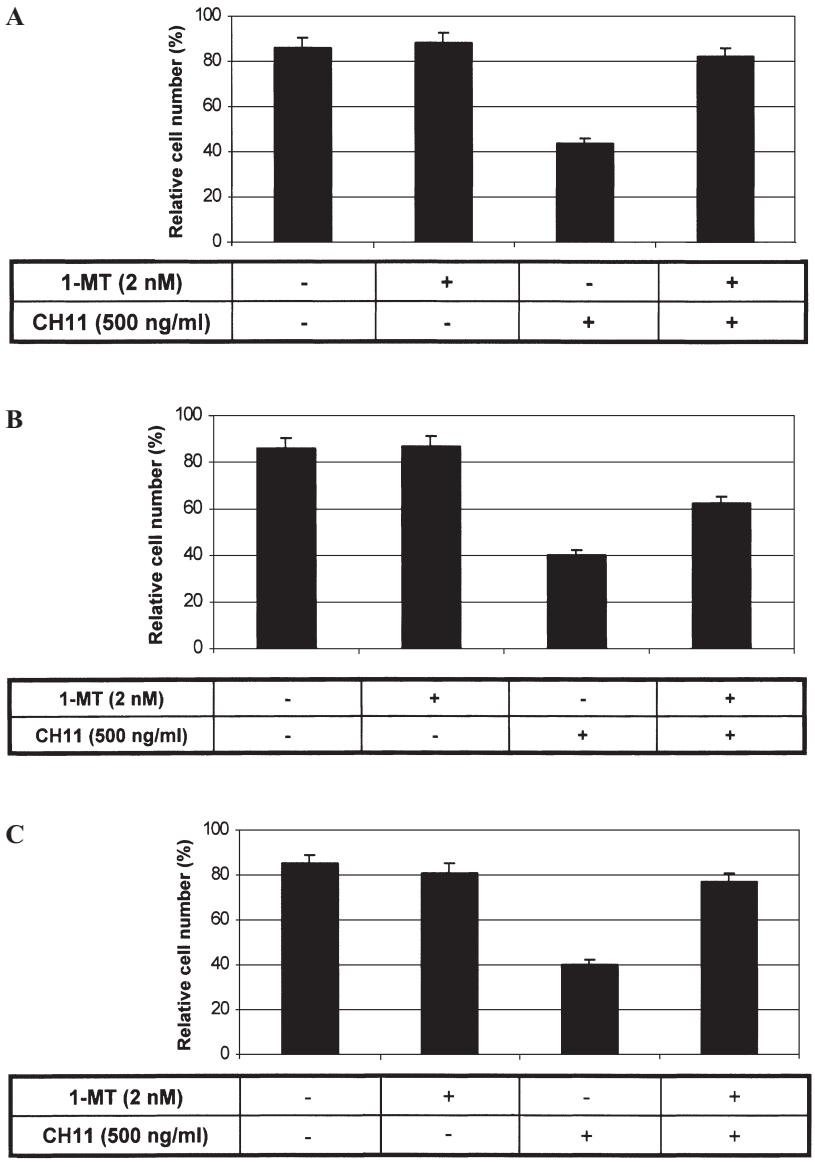


Figure 7. Pharmacological inhibition of IDO confers resistance to Fas-mediated cell death. Pretreatment with 1-MT, an inhibitor of IDO, prior to exposure to CH11 induced resistance to Fas-mediated cell death in HepG2 (*A*), A375 (*B*) and CLS-354 (*C*).

tance to Fas-mediated apoptosis in all tested cells. The identified fragments tagged at least 11 independent genes including APR-1, APR-2 and IDO, the inactivation of which generated resistance to Fas-induced apoptosis, illustrating the complexity of this process. In addition, 6 clones of the isolated fragments were unable to confer resistance in A375 cells, an important melanoma cell line, in contrast to HepG2 and CLS-354 cells where resistance to Fas-induced apoptosis was induced. Moreover, 9 clones of the isolated fragments tagging 6 genes whose inactivation generated resistance to Fas-induced apoptosis in HepG2 and A375 cells were unable to confer resistance in CLS-354 cells. Approximately 25% of Fas-resistant clones contained two or three different fragments. Whether these fragments are functionally additive will have to be examined.

The results of the present study demonstrate that antisense selection from episomal libraries of randomized cDNA fragments provides an efficient approach to identify functional genes. The same randomized library can be used to select antisense RNA segments inducing phenotypic changes in different cell types as we have shown using the HepG2 cDNA library to isolate antisense RNA segments that universally conferred resistance to Fas in different tumor cell types. The randomization of the cDNA population not only enables the cloning of cDNA sequences expressed at very low levels, as in the case of APR-1, -2 or IDO, but also provides insight into the mol-

ecular mechanisms regulating resistance to Fas-mediated apoptosis in heterologous cell types.

APR-1 and -2 are members of a protein family that were identified during all-trans retinoic-acid-induced apoptosis of acute promyelocytic leukemia cell lines [13]. The identification of APR-1 and -2 as the products of genes inhibited by their antisense RNAs suggests a potential role of these proteins in Fas-mediated apoptosis and confirms other reported data suggesting their role in the regulation of apoptosis [13].

IDO is a rate-limiting enzyme in the catabolism of tryptophan, expressed in a series of human and animal tissues, particularly in lymphoid organs and placenta [14]. In addition to its regulatory effects on T cells [15], different mechanisms lead to IDO-dependent immune regulation. These include induction of selective apoptosis of thymocytes and T cells in vitro [16, 17]. Apart from low-level expression in healthy individuals, enzyme production markedly increases during infection or inflammation and can be induced by lipopolysaccharide, cytokines, or other agents [18]. Several studies have shown that enhanced IDO activity is associated with inhibited proliferation of viruses [13] as well as with decreased proliferation of tumor cells [19].

Our selection strategy not only can be used to investigate previously unknown gene functions but also to identify functional antisense RNA segments of the corresponding gene leading to gene suppression. The antisense RNA segment approach is therefore a highly versatile tool for the analysis of biological processes in different tumor cells as well as for the identification of functional genes that may be clinically relevant for tumor therapy

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