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Advances in Brief

A Novel Candidate Tumor Suppressor, *ING1*, Is Involved in the Regulation of Apoptosis[1]

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Abstract

We have recently cloned a novel growth inhibitor and candidate tumor suppressor called p33^{ING1} (I. Garkavtsev *et al.*, Nature Genet., *14:* 415–420, 1996). Because some tumor suppressors participate in the regulation of apoptosis, we hypothesized that the *ING1* gene may also play a role in this process. Our results show that p33^{ING1} levels increase upon the induction of apoptosis in P19 teratocarcinoma cells by serum deprivation. Elevated expression of *ING1* in P19 and rodent fibroblast cells containing a tetracycline-controlled human c-*myc* gene enhanced the extent of serum starvation-induced apoptosis. This suggests that the pathway by which *ING1* modulates cell death is synergistic with Myc-dependent apoptosis. Conversely, constitutive expression of an antisense construct of *ING1* conferred protection against apoptosis in these cells. These data support the idea that loss of proper *ING1* function may facilitate tumorigenesis, in part, by reducing the cell's sensitivity to apoptosis.

Introduction

Programmed cell death, or apoptosis, is characterized by distinct morphological changes such as chromatin condensation, nuclear fragmentation, cytoplasmic blebbing, and DNA fragmentation (1). Many diverse substances and growth conditions have been identified that induce apoptosis, including irradiation, antigen recognition, cytotoxic drugs, glucocorticoids, and withdrawal of serum growth and survival factors (1–3). A common feature of many of these response pathways is the requirement for p53 tumor suppressor function (4). Loss of p53 function results in inappropriate cell cycle progression, uncontrolled growth, and tumorigenesis, and *p53* is the most frequently mutated gene in human tumors (5, 6). Reintroduction of p53 in deficient cells can induce either growth arrest (7–9) or apoptosis (10), depending upon cellular context. Some apoptotic events do not require p53, however, including glucocorticoid-mediated apoptosis of thymocytes (4, 11) and the developmentally programmed death of some embryonic cells (12, 13).

We have recently isolated *ING1*, a novel candidate tumor suppressor gene, using a method that combined subtractive hybridization of cDNAs from normal and cancerous cells with an *in vivo* selection assay (14). Because overexpression of the *ING1* gene efficiently arrests cells in the G_0/G_1 phase of the cell cycle, as does p53, we speculated that p33^{ING1} might be similarly involved in modulating apoptosis.

We therefore tested the potential of *ING1* to modulate serum starvation-induced apoptosis in P19 teratocarcinoma cells and in rodent fibroblast cell lines that conditionally overexpress a native human c-*myc* protein.[3] We demonstrate that overexpression of the p33^{ING1} protein confers sensitivity to apoptosis in these cells, whereas decreasing *ING1* expression using an antisense construct protects them from apoptosis. We therefore suggest that functional loss of *ING1* may contribute to tumorigenesis by diminishing a cell's capacity for apoptosis.

Materials and Methods

Cell Lines and Culture. P19 mouse teratocarcinoma cells were grown in α-MEM with 10% FCS (Life Technologies, Inc., Burlington, ON, Canada). For serum starvation experiments, the cells were washed twice in Ca^{2+}/Mg^{2+}-free PBS [137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4)], and serum-free α-MEM (Life Technologies, Inc.) was added. Rodent fibroblasts, either NIH 3T3- or rat 1-derived cells containing a tetracycline-controlled human c-*myc* gene (tet-myc cells)[3] were maintained in high-glucose DMEM (Life Technologies, Inc.) supplemented with 10% FCS and 2 μg/ml tetracycline (Sigma, St. Louis, MO) to repress premature human c-*myc* gene repression. The cells were treated as described previously[3] to induce c-*myc* expression and apoptosis. Briefly, this involves washing out the tetracycline inhibitor, elevating c-*myc* expression by up to 100-fold, and then transferring cells to medium without serum in which apoptosis rapidly ensues.

Retroviral Infection. Retroviral infection of target cells was used to introduce *ING1* expression constructs because we have previously demonstrated that ING1 protein seems to block entry into S-phase of the cell cycle (14), thereby precluding the use of standard drug-resistance selection methods. Retroviral infection also has a higher efficiency than standard calcium phosphate transfection procedures. The retroviral vector, pLNCX (15), containing sense, antisense (nucleotides 942-1124 of the *ING1* cDNA; Ref. 14), or vector alone was transfected into a highly efficient BOSC23 ecotropic virus-packaging cell line. The BOSC23 supernatant was then used to infect ecotropic producer cell lines. The target P19 and murine NIH 3T3 tet-myc cells were plated at 10^4 cells/10-cm plate and infected with undiluted viral supernatant from ecotropic producer cells. Infection efficiency was determined to be greater than 90% for NIH 3T3 tet-myc cells using a retroviral β-galactosidase construct. A lower efficiency (about 60%) was obtained from the P19 cells because these cells were prone to clumping.

Protein Preparation and Immunoblot Analyses. Nuclear proteins were isolated at the time points indicated and immunoblotted as described.[3]

Viability and Apoptosis Assays. Cell viability was assessed using a trypan blue dye exclusion assay. Cell suspensions were mixed 4:1 with 0.5% trypan blue:saline solution (Life Technologies, Inc.). The cells were incubated at room temperature for 5 min and counted with a hemacytometer.

DNA laddering was assessed using the method of Smith *et al.* (16). Tet-myc cells were plated at equal densities as described above. At 72 h after exposure to 0.1% FCS, DNA was isolated from the floating cells on a per-plate basis. Equal volumes of lysate were run on a 2% agarose gel and stained with ethidium bromide.

Indirect Immunofluorescence. Tet-myc cells containing the retroviral constructs were seeded at a density of approximately 10^4 cells/cm^2 on glass coverslips and grown at 37°C for 48 h before fixation and processing as described (17).

Microinjection Assays. Rat tet-myc cells were seeded on coverslips as described above and injected with about 25 μg/ml GST-p33-*ING1* protein or

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[3] C. C. Helbing, C. L. Wellington, M. Gogela-Spehar, T. Cheng, G. G. Pinchbeck, and R. N. Johnston. Growth factor deprivation activates cdk2 independently of c-*myc*-induced apoptosis, submitted for publication.

[4] The abbreviations used are: GST, glutathione *S*-transferase; CMV, cytomegalovirus.

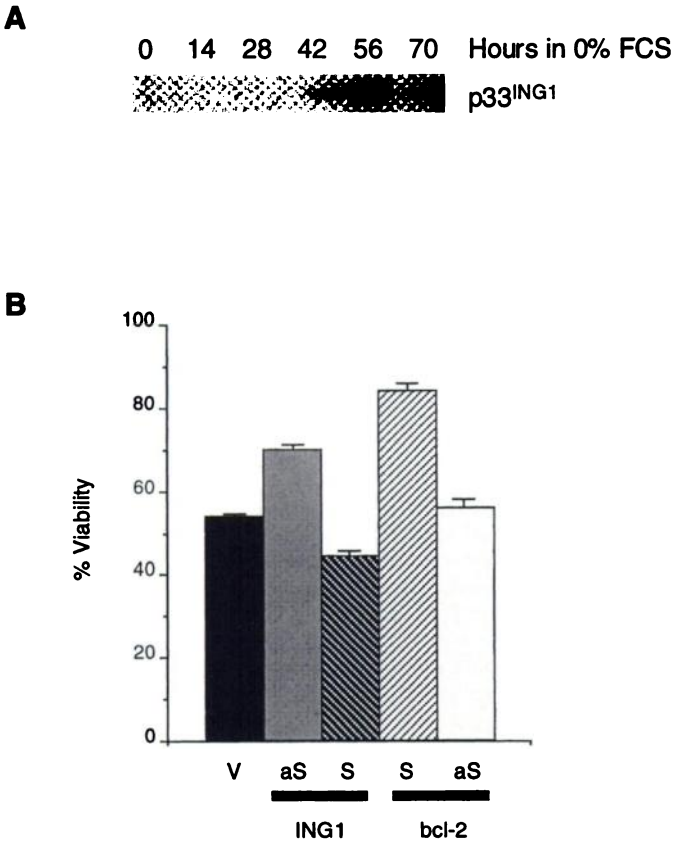


Fig. 1. *ING1* protein levels increase during serum starvation-induced apoptosis of P19 teratocarcinoma cells. *A*, P19 teratocarcinoma cells were serum-starved to induce apoptosis, and total protein was isolated at various times as indicated. Equal quantities of protein homogenates were loaded on SDS-polyacrylamide gels and Western blotted with a polyclonal antibody specific for *ING1*. *B*, trypan blue dye exclusion assay of serum-starved P19 cells infected with retroviral constructs expressing antisense *ING1* (*aS*), sense *ING1* (*S*), *bcl-2* sense (*S-bcl-2*), *bcl-2* antisense (*aS-bcl-2*), and vector-only (*V*) constructs. Cells were serum-starved for 72 h before assessing viability. The data shown are compiled from several independent experiments.

GST protein or with 25 μg/ml CMV-driven *ING1* expression construct or vector alone (18). After allowing the cells to recover for 4–6 h, the coverslips were washed twice with PBS, and the cells were serum-starved as described previously. Injected cells were identified through coinjection and subsequent immunostaining of a coinjected nonspecific antibody.

Results and Discussion

Indications that *ING1* may be involved in the modulation of apoptosis came from: (*a*) the conspicuous presence of *ING1* in regressing tail and its absence from growing hind limbs of metamorphosing *Xenopus* tadpoles;[5] and (*b*) the serum deprivation-induced elevation of *ING1* levels in P19 teratocarcinoma cells that correlated with the induction of apoptosis within 48–72 h (Fig. 1*A*; Ref. 19). To establish the effect of *ING1* expression on apoptosis, we infected P19 cells with sense (*S*), antisense (*aS*), and retroviral vector alone (*V*) and examined cell viability 72 h after serum withdrawal. As shown in Fig. 1*B*, the antisense ING1 construct conferred a moderate level of protection against death (70% viable) compared to that conferred by a retroviral construct constitutively expressing bcl-2 (84% viable). Conversely, P19 cells expressing the sense *ING1* construct exhibited greater death (44% viable) than the vector only (54% viable) or antisense bcl-2 controls (56% viable), suggesting that *ING1* confers cellular susceptibility to death induced by serum starvation. These numbers represent a minimal estimate of the effects of *ING1* and bcl-2 due to the modest transfection efficiency of P19 cells as outlined in "Materials and Methods."

Because the infection efficiency was low for P19 cells, we turned to another model system of apoptosis developed in our laboratory,[3] that of rodent fibroblasts (rat 1 and NIH 3T3) containing a tetracycline-controlled human c-*myc* gene (tet-myc cells). These tet-myc cells are maintained in 2 μg/ml tetracycline to repress the expression of a stably integrated human c-*myc* gene under the control of a minimal promoter and tetracycline operator sequences. Removal of tetracycline from the medium results in the rapid accumulation of human c-Myc protein (Fig. 2*A*) and subsequent apoptosis. The advantage of such a system is that control and experimental cells possess an identical genetic background, and the potential for cellular adaptation to constitutive c-*myc* overexpression is minimized. Because these cells form a monolayer, transfection efficiencies of greater than 90% are obtainable. The same *ING1* retroviral constructs as described previously (antisense, sense, and vector alone) were used on the NIH 3T3 tet-myc cells, and similar $p33^{ING1}$ expression levels were observed (Fig. 2, *B–D*) with localization of the protein to the nucleus (14, 16).

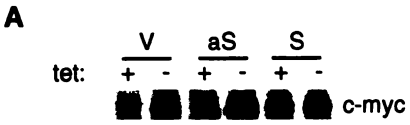
When myc was induced in the NIH 3T3 tet-myc cells by tetracycline withdrawal, and the cells were then serum-starved for 72 h, they showed a viability of 65% compared with controls in which *myc* was not induced (Fig. 3*A*). Apoptosis is minimized in cells coexpressing the antisense *ING1* construct, with only 5% of cells dying upon serum withdrawal. Cells expressing ING1 protein alone show 70% viability after serum starvation, and apoptosis is magnified when c-*myc* is also expressed (40% viable). These cells exhibited several hallmarks of apoptosis including shrinkage, loss of substrate adhesion, and chromatin condensation. In addition, internucleosomal DNA fragmentation is greatly enhanced in tet-myc cells expressing $p33^{ING1}$ compared to vector only and antisense *ING1* or *bcl-2*-expressing tet-myc cells (Fig. 3*B*).

To confirm that *ING1* enhances apoptosis during serum starvation, we microinjected $p33^{ING1}$ or a CMV-*ING1* expression construct into rat tet-myc fibroblasts and counted the number of remaining injected cells at various times after serum deprivation. Because the history of each injected cell could be followed, a more dramatic effect of *ING1* expression compared to the previous experimental approaches was apparent (45% viable at 24 h; Fig. 3*C*), with a further decrease in surviving cells when c-*myc* and *ING1* were coexpressed (9% viable at 24 h). These results support the data obtained with both the P19 and NIH 3T3 tet-myc cells and show that $p33^{ING1}$ is involved in regulation of apoptosis in a manner that is synergistic with the action of *myc*.

It therefore seems that in at least three independent cell lines, *ING1* expression confers an increased susceptibility to death upon serum starvation. Conversely, decreasing endogenous *ING1* levels afforded some protection against cell death. *ING1* markedly influences the outcome of c-*myc*-induced apoptosis and may therefore participate in guiding the response pathway whereby c-*myc* overexpression activates either apoptosis or tumorigenesis. Thus, a loss of $p33^{ING1}$ or its function seems to have similar consequences to those observed for p53 (2, 20). Because *ING1* seems to be important in the control of the G_1 to S-phase transition (14), it is possible that *ING1* could modulate or be modulated by p53. Conversely, *ING1* could act independently, perhaps providing an activity in which p53-independent mechanisms are at work. We are currently investigating these possibilities.

Several pieces of evidence suggest that *ING1* loss is of important biological consequence. Northern blot and reverse transcription-PCR analyses reveal a marked decrease in *ING1* RNA expression in a wide

[5] C. C. Helbing, K. Riabowol, R. N. Johnston, and I. Garkavtsev, Isolation and characterization of an *ING1* gene in *Xenopus laevis*, manuscript in preparation.



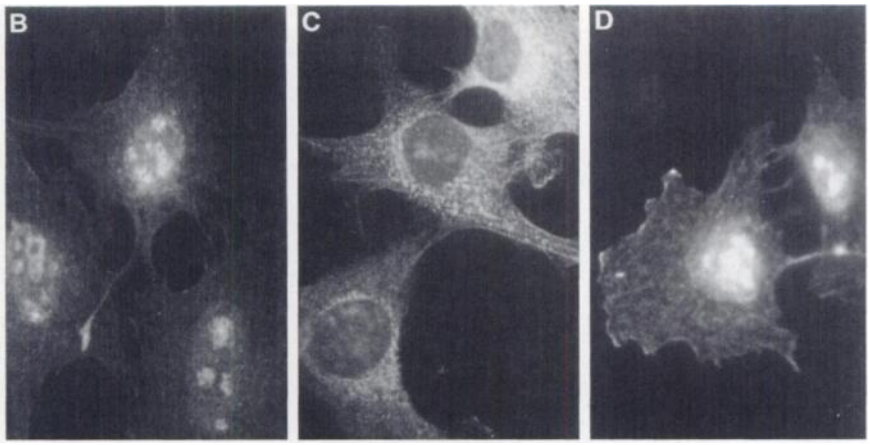


Fig. 2. Characterization of human c-*myc* and *ING1* expression in NIH 3T3 tet-myc cells. *A*, NIH 3T3 tet-myc cells were retrovirally infected with constructs containing vector only (*V*), antisense (*aS*), and sense (*S*) *ING1* constructs grown in the presence (+) or absence (−) of tetracycline. Removal of tetracycline from the medium results in a substantial accumulation of human c-*myc* protein as detected by a 1–9E10 mouse monoclonal antibody on Western blots of isolated nuclear proteins. Constitutive retroviral expression of *ING1* reveals that the $p33^{ING1}$ protein is exclusively found in the nucleus through immunohistochemical analyses of tet-myc cells containing vector only (*B*), antisense (*C*), and sense (*D*) *ING1* constructs. Polyclonal anti-*ING1* antibody (14, 21) was used. Note the conspicuous absence of nuclear staining in *C*, which was overexposed to reveal background cytoplasmic fluorescence.

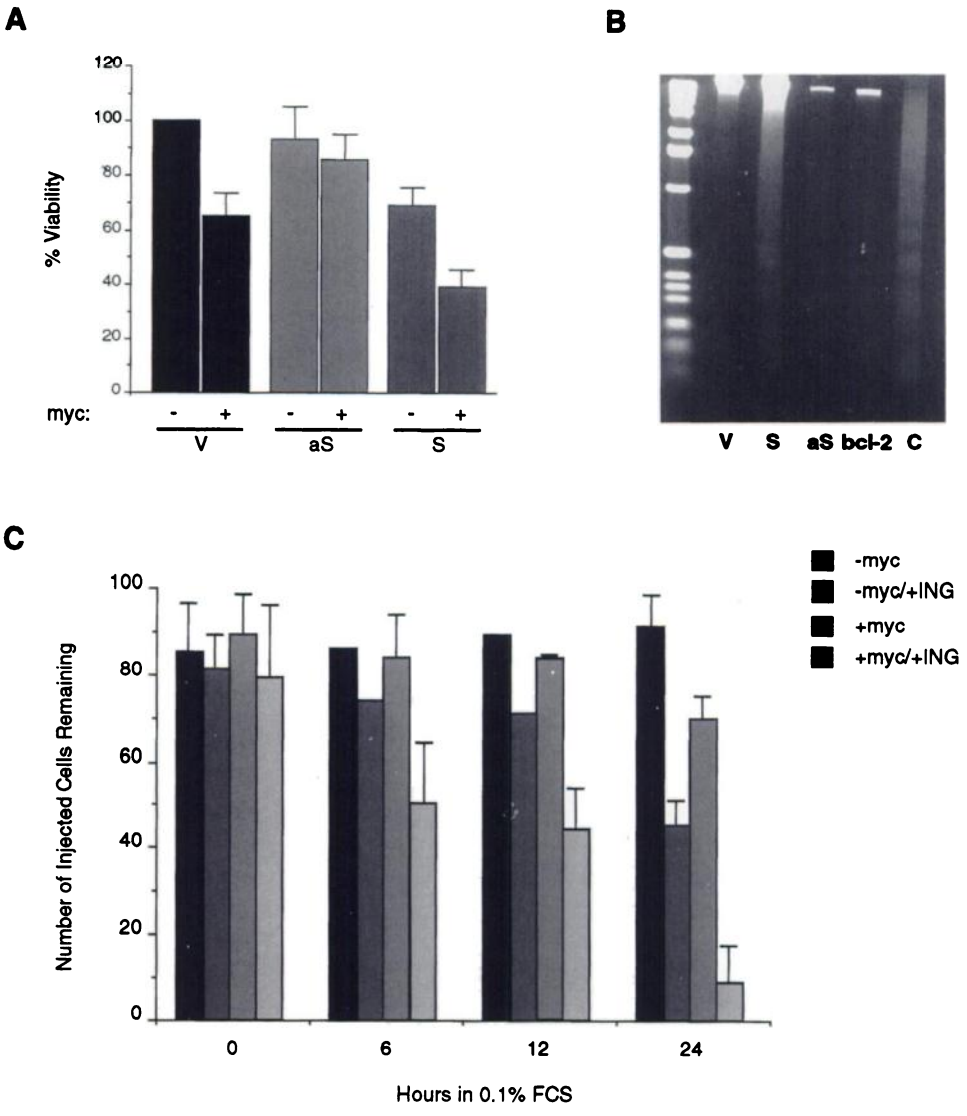


Fig. 3. Expression of *ING1* enhances c-*myc*-induced apoptosis. *A*, cell viability was determined using a trypan blue dye exclusion assay on NIH 3T3 tet-myc cells containing antisense (*aS*) or sense (*S*) *ING1* or vector-only (*V*) constructs after 72 h in 0.1% FCS in the presence (−myc) or absence (+myc) of tetracycline. The data show the results of three independent experiments and were normalized to vector-only cells not expressing c-myc. *B*, tet-myc cells expressing sense *ING1* (*S*) show a greater amount of DNA laddering than antisense (*aS*), vector only (*V*), and *bcl-2*-overexpressing tet-myc cells. DNA was isolated from the floating cells on a per-plate basis as described in "Materials and Methods" and separated on a 2% agarose gel. A positive control (*C*) from rat tet-myc fibroblasts undergoing apoptosis was also included for comparison. *C*, microinjection of GST-$p33^{ING1}$ protein or a CMV-*ING1* construct affects cell viability. One hundred rat tet-myc cells were injected with control GST fusion protein or CMV construct alone or with GST-$p33^{ING1}$ protein or CMV-*ING1* constructs (+*ING1*) in the presence (−*myc*) or absence (+*myc*) of tetracycline. After a brief recovery period, the injected cells were serum-starved in the presence (−*myc*) or absence (+*myc*) of tetracycline. Surviving cells were identified by immunostaining of a coinjected nonspecific antibody and counted at the indicated time points.

variety of tumor cell lines and primary tumors, and in addition, several tumor cells show mutations in the *ING1* gene (Ref. 14).[6] We therefore suggest that alterations in the proper functioning of $p33^{ING1}$ may contribute to tumorigenesis by rendering cells refractory to normal apoptotic pathways.

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