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Ref-1 regulates the transactivation and pro-apoptotic functions of p53 *in vivo*

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Ref-1 is a multifunctional protein that stimulates DNA binding by a number of transcription factors and serves as the abasic (A/P) endonuclease in base excision repair. Ref-1 was discovered to be a potent activator of p53 DNA binding *in vitro*. To address the physiological significance of the effects of Ref-1 on p53, we have analyzed its role in regulating p53 function *in vivo*. We found that Ref-1 over-expression enhances the ability of p53 to transactivate a number of p53 target promoters and increases the ability of p53 to stimulate endogenous p21 and cyclin G expression. Additionally, it was observed that Ref-1 associates with p53 *in vivo* and *in vitro*. Importantly, downregulation of Ref-1 (by antisense) causes a marked reduction in p53 induction of p21 mRNA and protein, as well as diminished ability of p53 to transactivate the p21 and Bax promoters. Moreover, Ref-1 levels are correlated with the extent of apoptosis induced by p53. Finally, we observed that Ref-1 cooperates with a DNA-damaging compound, camptothecin, to stimulate the transcriptional activity of p53. Together these data indicate that Ref-1 is a key cellular regulator of p53.

Keywords: apoptosis/DNA damage/p21/p53/Ref-1

Introduction

p53 plays a critical role in the response of cells to sources of stress such as DNA damage or hypoxia (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). Its ability to induce cell cycle arrest and apoptosis is due in part to its function as a sequence-specific transcriptional activator. By recognition of response elements within promoters that conform to the consensus sequence (5′-RRRCA/TT/AGYYY-3′)$_2$ (el-Deiry *et al.*, 1992) p53 can transactivate a number of target genes including, among many others, *p21*, *Bax* and *cyclin G* (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). p21 upregulation leads to cell cycle arrest by inhibiting cyclin-dependent kinase function (el-Deiry, 1993; Xiong *et al.*, 1993; Dulic *et al.*, 1994; Brugarolas *et al.*, 1995). Bax induces apoptosis by a still unknown mechanism, probably involving interaction with other members of the Bax/Bcl2 family (Oltvai *et al.*, 1993; Yin *et al.*, 1994; Chao and Korsmeyer, 1998), which may modify the mitochondrial ionic potential (Gross *et al.*, 1998; Matsuyama *et al.*, 1998). Cyclin G has been less well characterized but recent studies indicate that it too has pro-apoptotic activity (Okamoto, 1999).

After stress signals there are at least two ways in which p53 is activated: its levels are increased via post-transcriptional mechanisms, and p53 protein is converted into a form that is active for DNA binding and transcriptional regulation (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). Several studies have suggested that multiple covalent modifications of the p53 protein are necessary to allow stabilization and/or activation of p53 (Giaccia and Kastan, 1998; Prives, 1998). After DNA damage, p53 is inducibly phosphorylated and acetylated at a number of sites within its N- and C-termini. Phosphorylation of sites within its N-terminus are likely to affect its interactions with its negative regulator, Mdm2, as well as with components of the general transcription machinery, while phosphorylation and acetylation of sites within its C-terminus have been shown to stimulate DNA binding by p53 *in vitro*.

Previously we identified Ref-1 as a novel non-covalent activator of p53 (Jayaraman *et al.*, 1997). It was observed that Ref-1, at sub-stoichiometric concentrations, is able to convert latent p53 into a form that is activated for DNA binding *in vitro*. We found that Ref-1 affects the DNA-binding activity of p53 through both redox-dependent and -independent mechanisms. In fact, DNA binding by p53 has been shown previously to be strongly dependent on its redox state. Treatment of p53 with either oxidizing agents or metal chelators renders the protein incapable of DNA binding, whereas reduction of the protein enhances its DNA-binding activity (Hainaut and Milner, 1993; Delphin *et al.*, 1994; Jayaraman *et al.*, 1997). Additionally, mutations in critical cysteine residues abolish or alter p53 DNA-binding activity (Rainwater *et al.*, 1995).

Ref-1, also known as APE-1/Hap-1/APEX, is a DNA-repair (A/P) endonuclease (Demple *et al.*, 1991; Robson and Hickson, 1991; Seki *et al.*, 1991) that plays a central role in the process of base excision repair (BER). It functions to cleave DNA 5′ to abasic sites, as well as to load DNA polymerase γ onto DNA (Bennett *et al.*, 1997). The role of Ref-1 in BER has been studied by reconstituting, *in vitro*, a BER system combining purified DNA polymerase γ, uracil-DNA-glycosylase, DNA ligase I and Ref-1 (Dianov and Lindahl, 1994; Nicholl *et al.*, 1997). Moreover, the crystal structure of Ref-1 shows strong homology with two other DNA nucleases, DNase I and *Escherichia coli* exonuclease III (Gorman *et al.*, 1997).

In addition to its roles in BER, Ref-1 was shown previously to activate the AP-1 dimeric *fos–jun* complex by changing the redox state of its DNA-binding domain (Xanthoudakis and Curran, 1992). Interestingly, Ref-1 cooperates with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to activate AP-1 optimally, and this effect appears to be mediated by a pathway involving thioredoxin reductase and thioredoxin (Hirota *et al.*, 1997). The solution structure of thioredoxin and a peptide derived from Ref-1

support the existence of a physical, as well as a functional interaction between Ref-1 and thioredoxin (Qin *et al.*, 1996). Ref-1 increases the binding or the activity of other transcription factors, such as NF-κB, Myb (Xanthoudakis and Curran, 1992) or HIF-1 (Huang *et al.*, 1996), and is also a cofactor that participates in negative gene regulation after increases in intracellular calcium levels (Okazaki *et al.*, 1994; Chung *et al.*, 1996).

It is of interest that Ref-1 is itself regulated by various stress signals and post-translational modifications. Hypoxia, radical oxidized species and DNA-damaging drugs were each reported to lead to increased expression of Ref-1 and/or its nuclear translocation (Yao *et al.*, 1994; Grosch *et al.*, 1998; Ramana *et al.*, 1998). Additionally, phosphorylation by casein kinase II abolishes the DNA-repair function of Ref-1, presumably by regulating its exonuclease activity (Yacoub *et al.*, 1997).

It is a mystery why Ref-1, which is central to a critical DNA-repair process, can also affect the functioning of several seemingly disparate transcriptional regulators. Whatever the significance of its different roles in cells may be, Ref-1 is critical for early development, as deletion of the Ref-1 gene is lethal at a very early stage of embryogenesis (e5.5) (Xanthoudakis *et al.*, 1996). Moreover, downregulation of Ref-1 expression in Hela cells decreases clonogenic cellular survival after DNA-damaging drug treatment or hypoxia (Walker *et al.*, 1994). Finally, mice that are heterozygous for Ref-1 and null for p53 (and the *XPG* gene) manifested accelerated skin cancer after UV treatment when compared with wild-type mice (Meira *et al.*, 1997).

The ability of Ref-1 to activate proteins that are involved in the cellular response to various stresses (i.e. AP-1, NFkB and HIF-1), suggests that Ref-1 may play a role in signaling processes that protect the cell. The finding that Ref-1 stimulates p53 DNA-binding activity *in vitro* supports this hypothesis. Therefore, we wished to assess the importance of Ref-1 for the regulation of p53 activity *in vivo* and to determine whether p53 and Ref-1 are associated in cells. By experimentally regulating the levels of Ref-1 *in vivo*, we document herein a role for Ref-1 in both the transcriptional and the pro-apoptotic functions of p53.

Results

Stress signals including hypoxia, oxidative damage and some DNA-damaging agents that induce p53, also augment cellular levels of Ref-1. It is thus possible that under certain conditions the amount of Ref-1 may be limiting for p53. To study how enhanced levels of Ref-1 may affect p53 function *in vivo*, we established a number of stable H1299 clonally-derived cell lines in which tetracycline controls the expression of hemagluttinin (HA)-tagged Ref-1 protein (HA Ref-1), and we analyzed the regulation of p53 transcriptional activity in these cells.

Three such clones (cl-R12, cl-R13 and cl-R14) inducibly expressed HA-tagged Ref-1 (Figure 1A) while no detectable HA Ref-1 protein was observed in the presence of tetracycline. When HA Ref-1 was induced there was a 3- to 5-fold increase in total Ref-1 protein (corresponding to the HA Ref-1 and the endogenous Ref-1 proteins) (Figure 1A). Cells from the clone cl-R14 were co-transfected with a vector expressing p53 along with various reporter constructs containing one of the following p53-responsive promoters, p21–luc, Bax–luc and GADD45–luc (Figure 1D, E and F, respectively). Induction of HA Ref-1 expression in clone cl-R14 enhanced the transcriptional activity of p53 ~2-fold towards the three different promoters. Similar augmentation of p53 activity was observed in the two other clones, cl-R12 and cl-R13, (data not shown), but not in a control cell line (cl-Ct) established with an empty parental vector (similar to that utilized to construct HA Ref-1) (Figure 1B). The increased Ref-1 protein levels had little or no effect on these reporter constructs in the absence of p53 expression, or on additional reporter genes containing CMV, SV40 or minimal c-*fos* promoters (Figure 1C, and data not shown).

The ability of Ref-1 to stimulate p53 transcriptional activation was further examined using a H1299 cell line (H24-14) containing a tetracycline-regulated p53, in which induction of p53 had been shown previously to increase p21 expression and arrest the cells in G_1 and G_2 (Chen *et al.*, 1996). In H24-14 cells transient over-expression of HA Ref-1 augmented, in a dose-dependent fashion, the activity of a p21 promoter reporter (p21–luc) when p53 expression was induced by removal of tetracycline (Figure 2A). The reduction in stimulation at the highest level of Ref-1 might reflect a more general inhibitory effect, because there was a slight reduction of the p21–luc promoter activity in the absence of p53 at this dosage of Ref-1 plasmid, although at lower doses there was no significant effect of Ref-1 over-expression in the absence of p53. Note that when HA Ref-1 protein was expressed, there was a small increase in p53 protein levels (Figure 2B). Similar effects were observed using a reporter gene containing the 20 base pair (bp) p53 DNA-binding element from the p21 promoter (el-Deiry, 1993) upstream of the minimal c-*fos* promoter (Figure 2C; p21 min–luc). In contrast, Ref-1 had no effect on the parental vector (–53 c-*fos* luc) that lacks the p21-binding site (Figure 2D).

It was important to determine whether increasing the cellular levels of Ref-1 can enhance the ability of p53 to induce endogenously-expressed target genes. To examine this, we exploited a construct (pHook; Invitrogen, CA) that allows isolation of transfected cells. Ref-1 cDNA was cloned into this vector, which co-expresses a chimeric protein containing the transmembrane region of the PDGF receptor fused to the variable region of an antibody directed against the phOX hapten (Figure 3A; Materials and methods). Cells transfected with the Ref-1 or control pHook constructs were isolated on phOX magnetic beads, lysed, and p21 mRNA (Figure 3B) or cyclin G mRNA (Figure 3C) levels were assessed by RT–PCR. Cyclophilin mRNA levels were used as a standard. As expected, a dramatic increase in p21 mRNA was observed after cells were induced to express p53. Moreover, when Ref-1 was over-expressed there was a further increase of ~40% in p21 mRNA (Figure 3B). We observed that H1299 cells contain detectable levels of cyclin G mRNA in the absence of p53 (Figure 3C). Induction of p53, however, led to a 2-fold increase in cyclin G mRNA that was further enhanced by 50–100% upon Ref-1 expression (Figure 3C). Taken together our results show that elevated levels of Ref-1 in cells lead to increased transactivation by p53 of

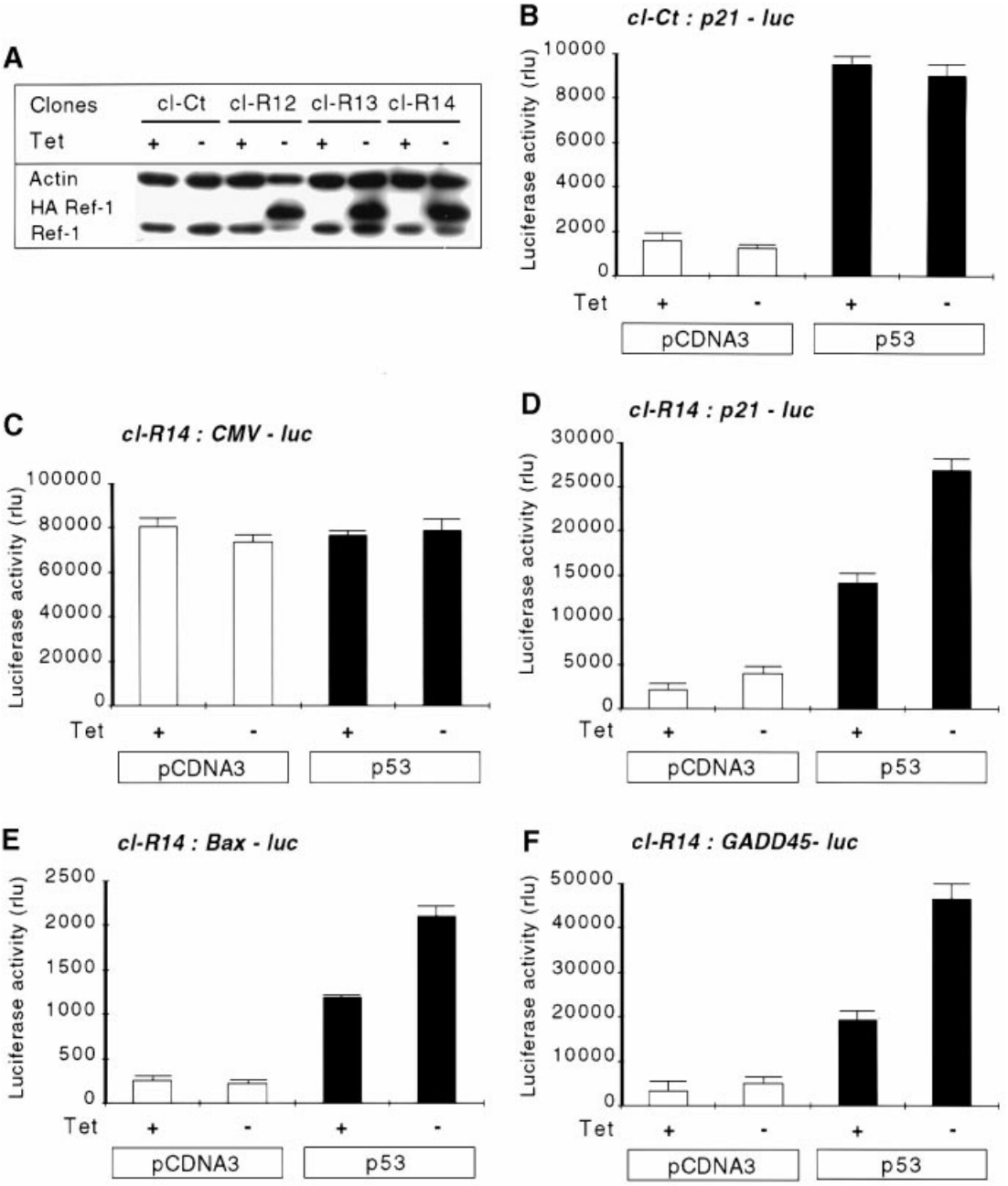


Fig. 1. Induction of Ref-1 expression increases p53-mediated transcription. (**A**) Western blot showing Ref-1 protein levels from three different clones of H1299 cells that express tetracycline-regulated HA-tagged Ref-1 (clones cl-R12, cl-R13 and cl-R14). As a control, clone cl-Ct was established with an empty tetracycline expression vector. An extract (30 μg) of cells growing in the presence of tetracycline (Tet +; 2 μg/ml) or 24 h after tetracycline removal (–) was used for the detection of both Ref-1 and actin. Endogenous Ref-1 (Ref-1) and ectopically expressed HA-tagged Ref-1 (HA Ref-1) are both indicated. (**B**) Cells from cl-Ct were co-transfected with pCMVp53wt and the p21–luc reporter. (C–F) Cells from the HA Ref-1-expressing clone cl-R14 were co-transfected with pCMVp53wt and either the CMV– (**C**), p21– (**D**), Bax– (**E**) or GADD45–luc (**F**) reporter genes. (B–F) Cells in 12-well plates were transfected with 400 ng of each reporter gene and pCMVp53wt. After 12 h, fresh medium was added with (+) or without (–) tetracycline and 24 h later, cells were harvested. Diagrams represent means in relative luminescence units (r.l.u.) and bars are standard deviations. Each diagram shows a representative experiment out of three, each done in triplicate.

a number of exogenous and endogenous p53-responsive promoters.

In vitro and in vivo association between p53 and Ref-1

Despite previous observations that Ref-1 stimulates p53 DNA binding *in vitro* and augments its ability to regulate transcription *in vivo* (Jayaraman *et al*., 1997; this study), there has been as yet no evidence documenting a physical interaction between the two proteins. To test this possibility we performed a 'Far-Western' assay in which Ref-1, Mdm2 and deleted Mdm2 (dl166) proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and renatured prior to incubation of the filter with immunopurified p53 protein (Figure 4A). After probing the filter with p53 antibodies, polyclonal antibodies (pAbs) 1801 and 421, we observed that p53 bound to renatured Ref-1 protein on the filter (Figure 4B). As controls, p53 bound to full-

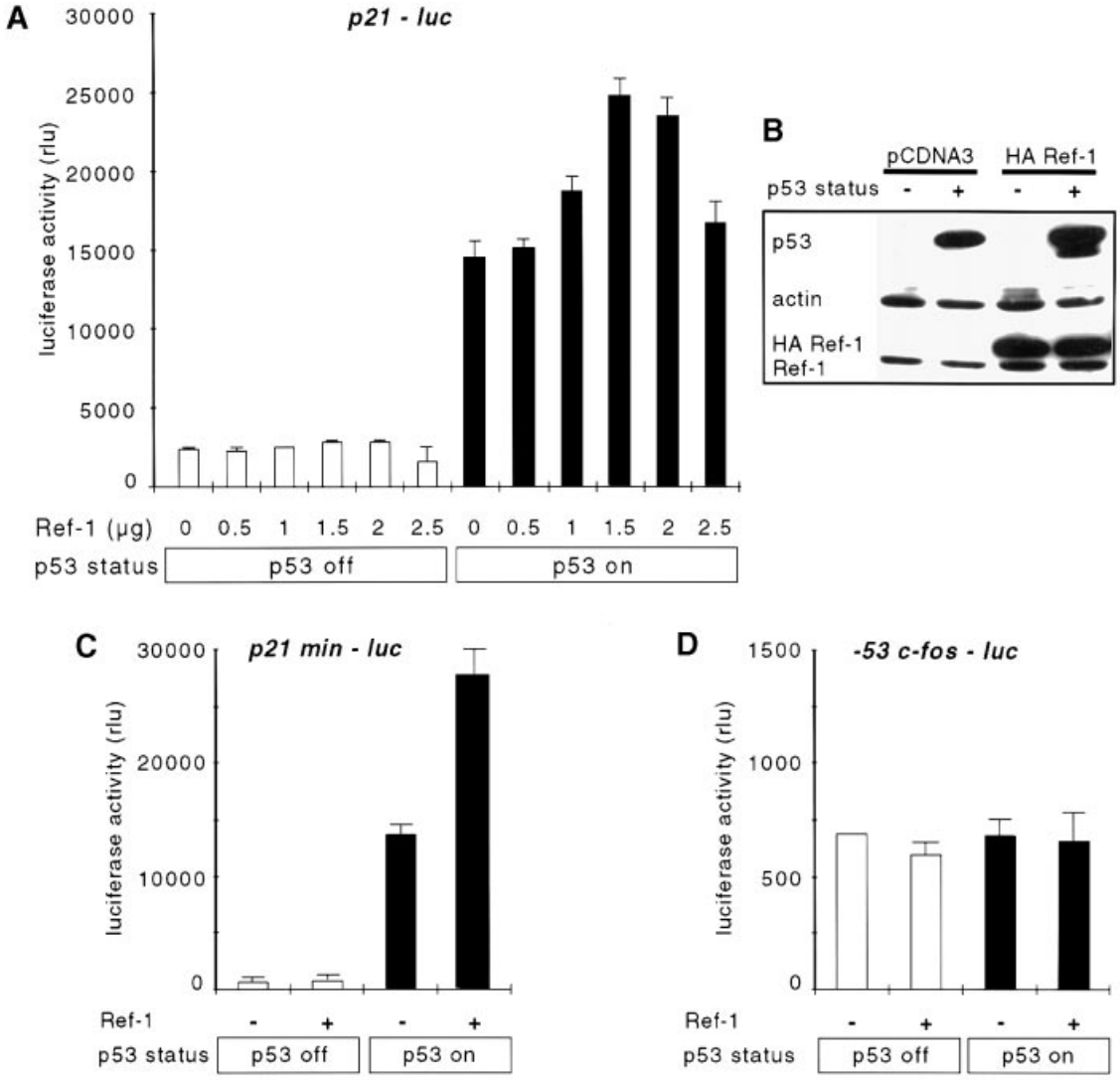


Fig. 2. Ectopic expression of Ref-1 in a p53-inducible cell line increases p53 transcriptional activity. (**A**) H24-14 cells expressing tetracycline-regulated p53 were cotransfected with the p21–luc reporter gene (500 ng) and increasing amounts of an HA Ref-1 expression vector at the quantities indicated (cells grown with tetracycline, p53 off; cells grown without tetracycline, p53 on). (**B**) Western blot showing p53, Ref-1 and actin protein levels. The gel was loaded with an extract (30 µg) of p53 tetracycline-regulated H1299 cells transfected with an HA Ref-1 expression vector (HA Ref-1) or empty pCDNA3 vector (pCDNA3) in the presence (+) or absence (–) of tetracycline. (**C** and **D**) H24-14 cells were cotransfected as described in Figure 1 with an HA Ref-1-expressing vector (1.5 µg) and either the p21 min–luc or the –53 c-*fos*–luc reporter construct (400 ng). Diagrams show means in relative luminescence units (r.l.u.) and bars are standard deviations. Each diagram shows a representative experiment out of two, each done in triplicate. In (A), results are normalized with the level of luciferase obtained with a CMV–luciferase reporter construct in each condition.

length Mdm2 but not the dl166 mutant Mdm2. Further evidence for the specificity of the interaction was derived from additional Far-Western experiments in which p53 bound to immobilized renatured Ref-1, but showed no detectable interactions with bovine serum albumin, glutathione *S*-transferase or several native standard molecular weight marker proteins (data not shown). Additionally, in a reciprocal Far-Western experiment, p53 immobilized and renatured on nitrocellulose was shown to bind to soluble Ref-1 protein (data not shown). Using this approach, we conclude that p53 and Ref-1 can directly and specifically interact with each other.

To address whether p53 and Ref-1 may exist in the same protein complex *in vivo*, the p53-inducible H24-14 cell line was used to perform 'IP-Western' analysis of cell extracts before and after induction of p53. Cell extracts were incubated with a mixture of p53 monoclonal antibodies (mAbs), and the resulting immunoprecipitate was resolved by SDS–PAGE, transferred to nitrocellulose, and probed for the presence of Ref-1 on a Western blot using a Ref-1 polyclonal antiserum (Figure 4C). When p53 was induced, we observed that it co-immunoprecipitated with Ref-1. The amount of total detectable Ref-1 that interacts with p53 was estimated to be ~5%, as indicated by comparison of the intensity of the bands present after IP-Western (Figure 4C, bottom left) with a direct Western blot in which 5% of the extracts were used for immunoprecipitation (Figure 4C, bottom right). Conversely, when Ref-1 was immunoprecipitated with the Ref-1 pAb, p53 was detected in the immune complex by probing with the same mixture of p53 mAbs (Figure 4D, left). Here too, we estimate that no more than 5% of p53 interacts with Ref-1 by comparing the levels obtained by direct Western blotting with 2.5% of the extract used for immuno-

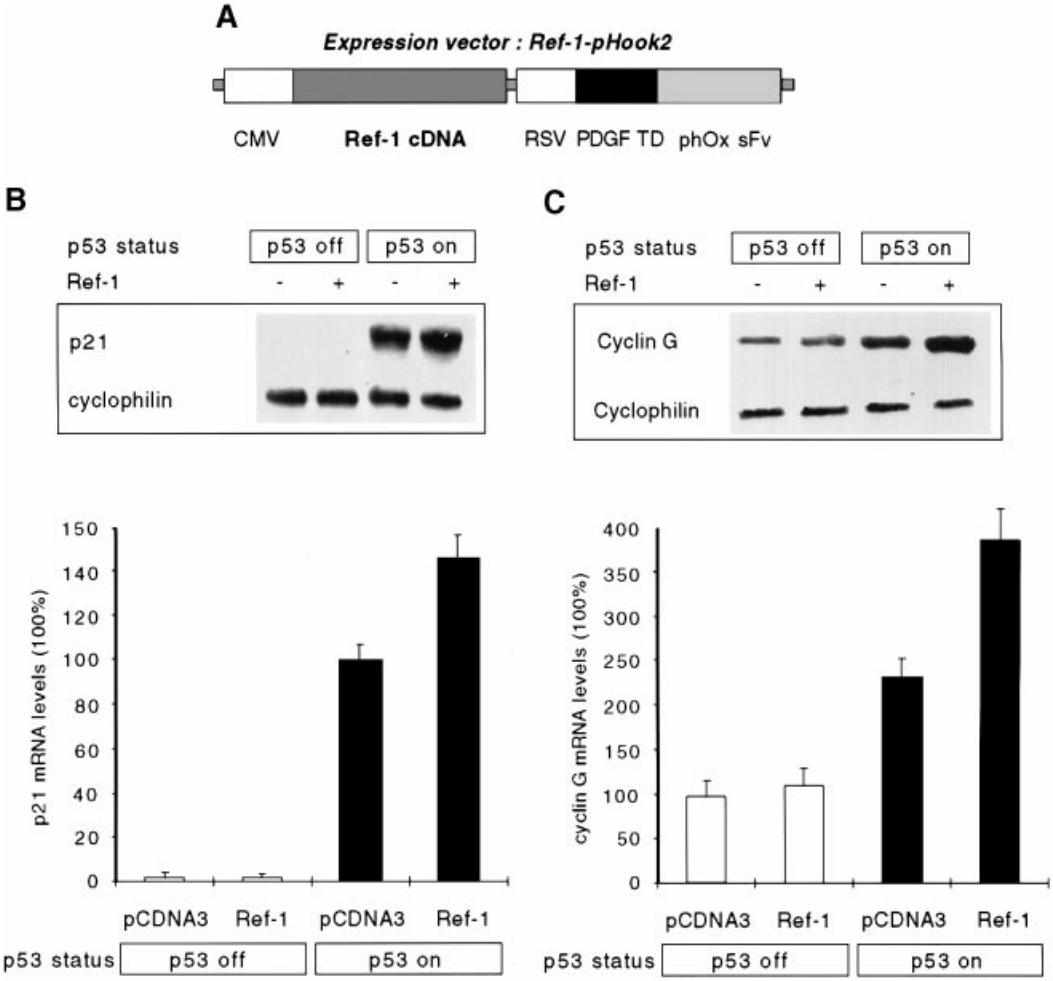


Fig. 3. Ectopic expression of Ref-1 in a p53-inducible cell line increases p53 induction of endogenous p21 and cyclin G mRNA levels. (**A**) Schematic representation of the pHook2-Ref-1 vector. Ref-1 cDNA is driven by the CMV promoter. The cDNA coding for a chimeric protein formed by the PDGF receptor transmembrane domain (PDGF TD) fused to the variable region of the antibody directed against the phOx apten (phOx sFv) is downstream of the Rous sarcoma virus early promoter (RSV). (**B** and **C**) H1299 p53-inducible cells were transfected with pHook2-Ref-1 vector (20 μg/10-cm plate) for 72 h and cells were further grown in the presence (p53 off) or absence (p53 on) of tetracycline for 12 h. p21 and cyclin G mRNA were amplified by RT–PCR and products were resolved on a 2% ethidium bromide-stained agarose gel [(B) and (C), upper panel]. Quantification of p21 and cyclin G mRNA shown in [(B) and (C), lower panel] was performed by an image analysis program (NIH image 1.39). Results were normalized with cyclophilin mRNA. Diagrams represent means in fold induction compared with cells transfected by a pCDNA3 vector with p53 off. Bars are standard deviations obtained from three individual experiments.

precipitation (Figure 4D, right). These experiments therefore suggest that a small proportion of the Ref-1 and p53 in H1299 cells are present in the same protein complex. Although it cannot be concluded that there is a direct interaction between p53 and Ref-1 *in vivo*, this possibility is supported by the results of the Far-Western assay.

Downregulation of Ref-1 decreases p53-induced p21 protein levels and p53 transcriptional activity

To address further the physiological importance of Ref-1 for p53 activity, an antisense approach was employed in order to reduce the expression of endogenous Ref-1 protein. The pHook expression vector described above, engineered to express a Ref-1 antisense RNA, was transiently transfected into the H1299 p53-inducible cell line, H24-14. In these cells p21 is detected only after p53 is induced by tetracycline withdrawal (Chen *et al.*, 1996; Figure 5A). Isolated cells were lysed, and p21, Ref-1, p53 and actin protein levels were assessed by Western blot analysis. With increasing amounts of the antisense Ref-1 vector, endogenous Ref-1 protein levels declined such that this protein was virtually undetectable at 2 μg of transfected vector (Figure 5A). Under these conditions, downregulation of Ref-1 was correlated with dramatically reduced endogenous p21 protein levels induced by p53. Note that even at the highest concentration of antisense vector, p53 and actin levels were only modestly affected, suggesting that the main effect of loss of Ref-1 was on the activity of p53.

Since p21 is regulated by p53 at the transcriptional level (el-Deiry, 1993), we also examined p21 mRNA levels in the H24-14 p53-inducible cell line under conditions of decreased Ref-1 protein (Figure 5B). p21 mRNA was detected by RT–PCR and corrected by comparison with the mRNA levels of the cyclophilin gene, which is not regulated by p53. As expected, induction of p53 expression strongly increased p21 mRNA levels. When Ref-1 expression was downregulated by Ref-1 antisense, however, induction of p21 mRNA levels by p53 was reduced by 50%, as estimated by quantitation of the p21 mRNA by

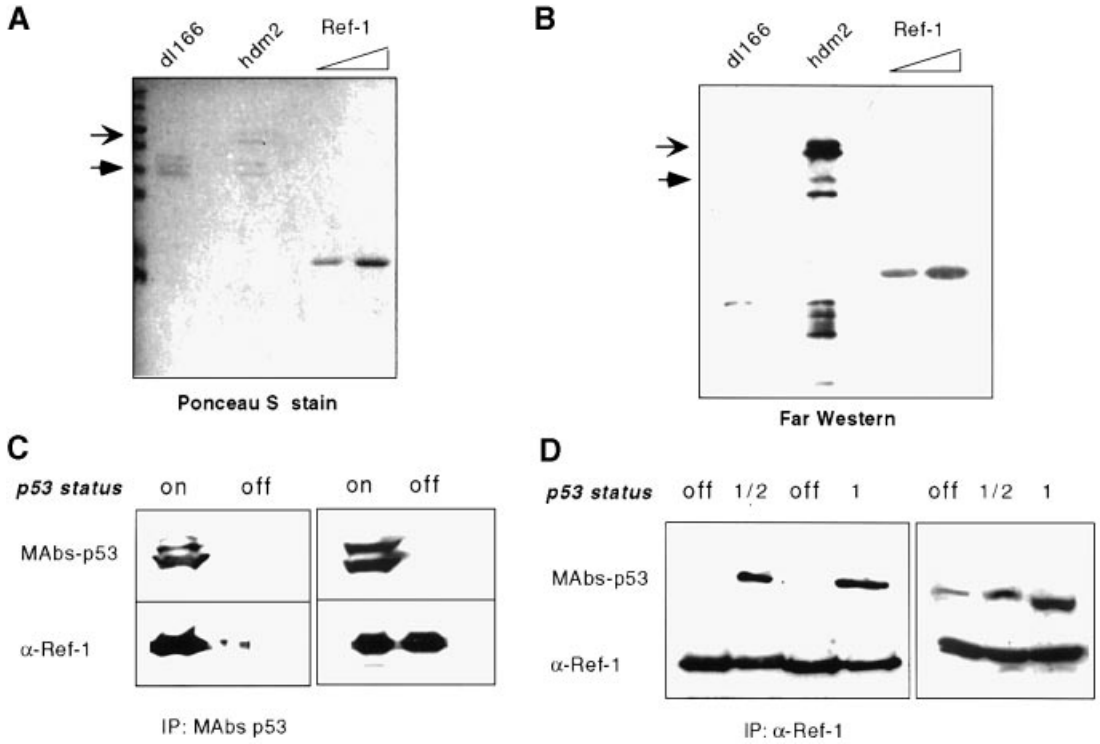


Fig. 4. Association of p53 and Ref-1 *in vitro* and *in vivo*. (**A** and **B**) Far-Western analysis. Ref-1 (1 and 2 μg) and wild-type and dl166 Mdm2 (1 μg each) proteins were resolved by SDS–PAGE and transferred to nitrocellulose. The membrane was first stained with Ponceau-S (A) and then incubated with purified p53 (1 μg in 5 ml) followed by detection using pAb 421 as primary antibody (B). (**C** and **D**) Co-immunoprecipitation of p53 and Ref-1 from extracts of H1299 cells. Cells were grown in the presence (p53 off) or absence (p53 on) of tetracycline for 24 h before harvesting and extraction. In (D) an intermediate amount of tetracycline was used such that only half the p53 protein (1/2) was induced compared with that seen after a complete removal of tetracycline (1). p53 protein was detected by a mixture of mAbs (mAb 1801, 421 and 240), and Ref-1 by a pAb (α-Ref-1). Extracts, 5% (C) and 2.5% (D) of the amount used for the immunoprecipitation, were incubated with the p53 antibody mixture (C) or the α-Ref-1 antibody (D). After resolution by SDS–PAGE, and transfer to nitrocellulose, filters were incubated with p53 antibody mixture or α-Ref-1 antibody. Positions of p53 and Ref-1 polypeptides are indicated.

densitometry (Figure 5C). In the absence of p53 protein, Ref-1 had no effect on p21 expression.

Further support for the likelihood that the reduction of p21 mRNA levels obtained when Ref-1 was diminished involves a decrease of p53 transcriptional activity, was derived from an experiment in which p21-promoter activity in the H1299 p53-inducible cell line was analyzed by using a reporter gene assay (Figure 6). While, in the presence of normal cellular levels of Ref-1, p53 strongly stimulated the activity of the transfected p21-promoter reporter gene (Figure 6A), the transcriptional activity of p53 was markedly reduced with increasing amounts of transfected Ref-1 antisense DNA. Under conditions where there was no detectable Ref-1 protein (e.g. Figure 5A), the stimulation of p21-promoter activity by p53 was reduced by 60%. The transcriptional activity driven by the CMV promoter (pCMV luc) was not affected by antisense Ref-1, indicating the specificity of Ref-1 down-regulation on p53 transcription. It is likely that the small reduction in activity of the p21–luc promoter in the presence of antisense Ref-1 reflects very minor leakiness of the tetracyline-regulated construct expressing p53. We further extended this analysis to the Bax promoter, observing that induction of this reporter by p53 was similarly reduced by downregulation of Ref-1 expression (Figure 6B).

Our data thus show that downregulation of Ref-1 decreases the ability of p53 to serve as a transcriptional activator *in vivo*. This effect is observed at three levels: (i) endogenous p21 protein levels; (ii) endogenous p21 mRNA levels; and (iii) exogenous p21-promoter activity. Moreover, decreased expression of Ref-1 reduces Bax-promoter activity, suggesting that Ref-1 has a more widespread negative effect on p53 function.

Ref-1 regulates the ability of p53 to induce apoptosis

The apoptotic function of p53 is considered to be an essential component of its function as a tumor suppressor. The significance of p53-mediated apoptosis is supported by a number of studies, including the fact that several tumor-derived p53 mutants, which are defective in induction of apoptosis, display selective transcriptional activity. These mutants are able to transactivate several p53-responsive promoters, with the exception of some genes such as *Bax* (Friedlander *et al.*, 1996; Rowan *et al.*, 1996; Flaman *et al.*, 1998; Ryan and Vousden, 1998). Our observation that Ref-1 levels are correlated with the extent to which p53 transactivates the Bax promoter (Figure 1C and 6B) suggested the possibility that Ref-1 might also regulate the pro-apoptotic function of p53. To test this, H1299 cells were transfected with wild-type p53 along with an expression vector that allows, via an inter-ribosomal expression sequence (IRES), the co-expression of both the green fluorescent protein (GFP) and either the sense or the antisense Ref-1 sequence (Figure 7A). After

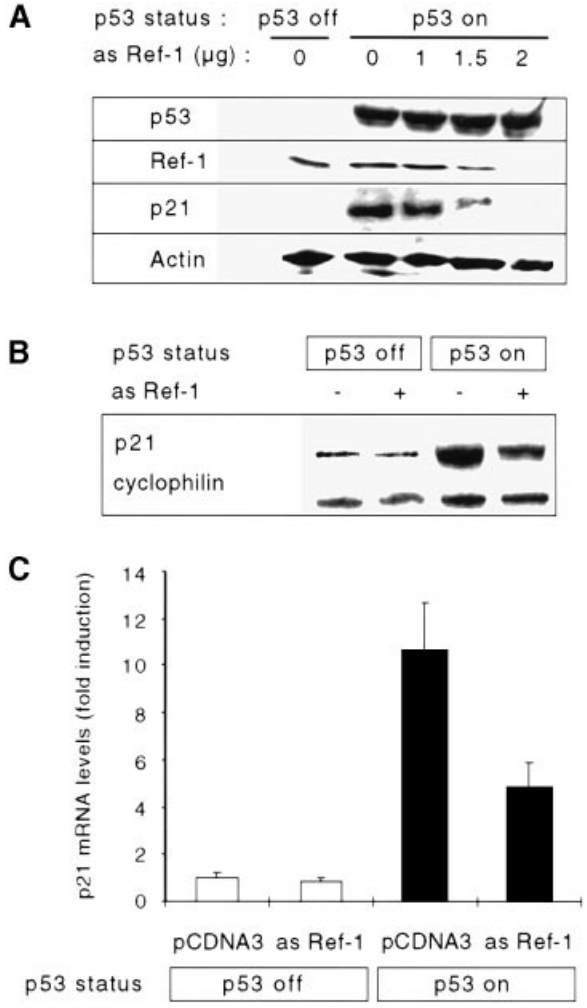


Fig. 5. Downregulation of Ref-1 decreases p53-induced p21 expression. (**A**) Protein levels in the H24-14 p53-inducible cell line after downregulation of Ref-1 expression. Cells were transfected with increasing amounts (0 to 20 μg DNA/10 cm well) of pHook2-Ref-1 antisense vector (as Ref-1) for 72 h, and then grown in the presence (p53 off) or absence (p53 on) of tetracycline for 12 h. Western blots were performed with 50 μg of cell extract, and p53, Ref-1, p21 and actin proteins were detected with appropriate antibodies. (**B** and **C**) H24-14 cells were transfected with the pHook2-Ref-1 antisense vector (20 μg/10-cm plate) and treated as described in (A). p21 mRNA was analyzed as described in Figure 3. Diagrams represent means in fold induction compared with cells transfected by a pCDNA3 vector with p53 off. Bars are standard deviations obtained from three individual experiments.

transfection (72 h), the morphology of transfected cells was analyzed. An example of the morphological changes induced in cells expressing p53 is shown in Figure 7B, right panel, and an example of cells expressing a control plasmid is shown in Figure 7B, left panel. p53 over-expression induced apoptotic bodies and membrane blebbing, which are characteristic of apoptotic cells (Hale *et al.*, 1996). By enumerating such morphologically distinguishable cells as a function of the total GFP-expressing cells it was possible to quantify the effects of Ref-1 on the ability of p53 to induce apoptosis (Figure 7C). As shown previously (Haupt *et al.*, 1995; Friedlander *et al.*, 1996; DiComo *et al.*, 1999), transient transfection of wild-type p53 induced apoptosis (see Figure 7C, where the ordinate represents the percentage of apoptotic cells divided by 500 GFP-stained cells per 10-cm plate). Co-expression of p53 with Ref-1 resulted in an increase of apoptotic cells to a level ~30% higher than that seen with p53 alone (Figure 7C). Moreover, reduced expression of Ref-1 (by antisense) decreased the number of apoptotic cells by 40%. It should be noted that up- or downregulation of Ref-1 in the absence of p53 expression did not appear to affect the morphology of the cells.

The effects of Ref-1 on p53-mediated apoptosis were confirmed by analyzing the fraction of cells with a sub-G_1 content of DNA obtained under the different conditions. H1299 cells were co-transfected with p53 expression vectors and either a sense or antisense Ref-1/GFP-expressing vector, as described in the Materials and methods section. Then, cells were fixed 72 h post-transfection, stained with propidium iodide (PI), and passed through a fluorescence activated cell sorter (FACS). GFP-stained cells were gated by comparing cells transfected with an empty vector with those transfected with the GFP-expressing vector. GFP-positive cells were analyzed for their DNA content, in order to quantify the sub-G_1 population when only GFP is expressed. The results observed under the different conditions are summarized in Figure 7D as the percentage of cells in the sub-G_1 fraction divided by the percentage of GFP-positive cells. We observed that over-expression of p53 alone in H1299 cells increased the sub-G_1 fraction from 3 to 19%. Co-expression of Ref-1 with p53 further increased the sub-G_1 fraction to 25.7%. In contrast, downregulation of Ref-1 expression reduced the sub-G_1 fraction to 11.5%. Therefore, our results show a strong correlation between Ref-1 expression and the extent of both transactivation and pro-apoptotic functions of p53.

Ref-1 cooperates with camptothecin to activate p53-mediated transcription

We observed previously that administration of the topoisomerase inhibitor camptothecin (CPT) facilitates p53-mediated apoptosis in H1299 cells (Chen *et al.*, 1996; our unpublished results). While the mechanism of such cooperation is not yet understood, it is important to note that the levels of p53 are not affected by CPT treatment in these cells (Chen *et al.*, 1996; and our unpublished data). We tested the hypothesis that Ref-1 and CPT may collaborate to stimulate p53-dependent transcription. The p53-responsive p21 min–luc reporter gene, with or without co-expression of a Ref-1 expressing plasmid, was transfected into cells that had been treated or not with CPT (Figure 8). Interestingly, despite the unaltered levels of p53 protein, p53-dependent transcription of the reporter gene was stimulated when cells were treated with CPT. Consistent with results described in Figures 1 and 2, Ref-1 over-expression also stimulated the p53-dependent activity of the reporter gene. Indeed, Ref-1 cooperated significantly with CPT to further induce p53 transcriptional activity.

To extend this observation, we analyzed the effect of altered Ref-1 expression in RKO cells whose endogenous wild-type p53 levels and activity are increased after DNA damage (Kessis *et al.*, 1993; Nelson and Kastan, 1994). The transcriptional activity of p53 was assessed using the minimal p21 promoter (p21 min–luc), transfected along with either a sense or an antisense Ref-1 vector. While

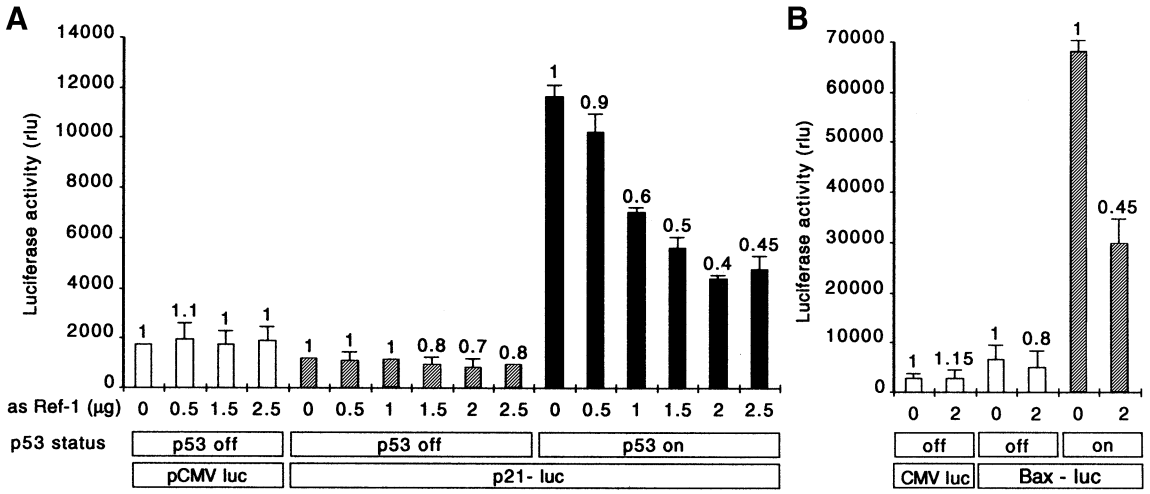


Fig. 6. Ref-1 downregulation reduces p53 stimulation of p21 and Bax promoters. (**A**) H24-14 cells were co-transfected with various amounts of pHook2-Ref-1 antisense vector (as Ref-1) along with different reporter genes containing the luciferase cDNA driven by either the p21 promoter [p21–luc; (A) right], the early promoter of the cytomegalovirus [pCMV–luc; (A) left], or the Bax promoter [Bax–luc; (**B**)]. Transfected cells were isolated and treated as described in Figure 5. Diagrams represent means in relative luminescence units (r.l.u.) and bars are standard deviations. Each diagram shows a representative experiment out of three, each done in triplicate. Numbers above each bar indicate fold induction relative to 0 μg of antisense Ref-1.

CPT treatment of RKO cells stimulated the activity of a p53-responsive element derived from the p21 promoter as expected (Figure 8B), Ref-1 over-expression further increased the effect of the inhibitor. Importantly, downregulation of Ref-1 expression (see insert in Figure 8C) led to a significant reduction of the p21 minimal promoter activity induced by CPT (Figure 8C). While it is not clear whether CPT directly affects Ref-1 function, or whether the two treatments work independently to augment p53 activity, our results show that the effect of a DNA-damaging agent on p53 is facilitated by Ref-1.

Discussion

Ref-1 was previously discovered to be a potent activator of p53 DNA-binding activity *in vitro* and to stimulate the ability of p53 to transactivate a p53 reporter gene in a transient transfection assay (Jayaraman *et al.*, 1997). Here, we have extended and validated these observations by demonstrating a physiological role for Ref-1 in regulation of p53 *in vivo*. We show that: (i) Ref-1 and p53 associate *in vivo* and *in vitro*; (ii) Ref-1 protein levels are correlated with corresponding changes in p53 transcriptional activation of a number of p53 target promoters; (iii) downregulation of Ref-1 expression leads to a decrease of endogenous p21 expression induced by p53; (iv) induction or downregulation of Ref-1 levels are correlated with the ability of p53 to induce apoptosis; and (v) Ref-1 cooperates with a DNA-damaging drug to stimulate p53 transcriptional activity.

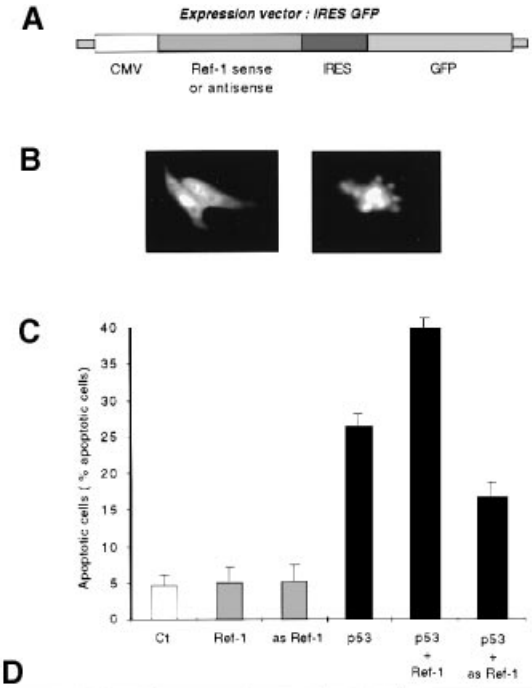
Ref-1 regulates the transcriptional activity of p53 in vivo

Over-expression of Ref-1, either by use of inducible cell lines or transient transfection, enhances the transactivation function of p53. While the effects observed were in the order of a 2-fold stimulation, this is still remarkable, since Ref-1 protein levels are rather high in cells. This suggests that, despite its relative abundance, Ref-1 is limiting under some conditions. It is significant that Ref-1 augments the ability of p53 to stimulate several reporters including ones containing Bax, GADD45 and p21 promoters (this study) and the cyclin G promoter (Jayaraman *et al.*, 1997). These results suggest that Ref-1 may affect the overall transcriptional activity of p53, although a more extensive panel of target genes still needs to be tested before we can reach that conclusion. Ref-1 stimulates p53 activity on a reporter construct containing the minimal p53 response element from the p21 promoter, suggesting that Ref-1's effects are mediated by a p53-binding site and are not likely to involve other elements in the more complex promoters used in the other reporter constructs.

Most strikingly, we found that downregulation of Ref-1 protein levels markedly impairs the ability of p53 to induce the expression of endogenous p21. However, p21 protein levels, mRNA levels and promoter activity were not affected to similar extents by Ref-1 downregulation. p21 protein levels were almost undetectable, in contrast to its mRNA levels or promoter activity, which were reduced by ~50%. This discrepancy could be due to differences in sensitivity between the assays. Alternatively, Ref-1 may affect p21 protein levels in a transcription-independent manner. It may be relevant that p21 expression is regulated by redox and that this involves post-transcriptional mechanisms (Esposito *et al.*, 1997). Downregulation of Ref-1 does not exclusively affect p21-promoter activity, as Bax expression is also reduced (Figure 6B), implying that Ref-1 is required for optimal p53 transcriptional activity involving its other target genes. The fact that similar inhibition was obtained on both promoters (~50–60%) is in agreement with this suggestion.

The ability of p53 to induce apoptosis is affected by Ref-1 levels

By using two different assays to quantify cell apoptosis, we were able to correlate the level of Ref-1 with the induction of cell death by p53. We observed that over-expression of Ref-1 increases the ability of p53 to induce



D

Plasmids	Ct	Ref-1	as Ref-1	p53	p53 +Ref-1	p53 +asRef-1
Sub G1 % total	1.9	2.42	2.1	1.78	1.81	2.04
Sub G1 % GFP	2.99	3.4	2.8	18.94	25.7	11.45

Fig. 7. Ref-1 regulates the ability of p53 to induce apoptosis. (**A**) Schematic representation of the bisistronic vector used for co-expressing Ref-1 or Ref-1 antisense and GFP. Ref-1 sequences (sense or antisense) and GFP cDNA are driven by the CMV promoter. The IRES is located between the Ref-1 sequence (sense or antisense) and the GFP cDNA. (**B**) p53 was co-expressed with Ref-1 or Ref-1 antisense in H1299 cells. The GFP bisistronic vector (IRES GFP) containing a Ref-1 sequence (sense or antisense) was transfected along with pCMVp53wt. Morphology of GFP-stained cells was analyzed under a microscope to detect the presence of apoptotic bodies. Representative examples are shown: co-expression of p53 and Ref-1 (right panel), cells transfected with an empty IRES GFP vector and an empty pCDNA3 vector (left panel). (**C**) Quantification of apoptosis in GFP-stained H1299 cells expressing p53 and Ref-1 or Ref-1 antisense (as Ref-1). Results are expressed as the percentage of cells displaying apoptotic morphology out of 500 GFP-stained cells in a 10-cm plate. Bars represent means from three individual plates with error bars shown. Each diagram shows a representative experiment out of three. (**D**) Detection of sub-G_1 fraction in GFP-positive cells by FACS analysis. The table shows the percentage of sub-G_1 cells in the total population of transfected cells (Sub-G_1 % total) and in GFP-stained cells (Sub-G_1 % GFP). Results are from one representative experiment out of two.

apoptosis, while downregulation of Ref-1 reduces this ability. The fact that Ref-1 downregulation does not abolish p53 induction of apoptosis may be explained by previous observations that there is a transcription-independent component of p53 function in apoptosis (Haupt *et al.*, 1995 and references therein), including in H1299 cells (Chen *et al.*, 1996). It was demonstrated previously that over-expression (Tomicic *et al.*, 1997) or downregulation (Walker *et al.*, 1994) of Ref-1 by itself does not seem to affect cell viability, conclusions that are supported by our observations. It remains to be established whether Ref-1 can also modulate the ability of p53 to induce cell cycle arrest (G_1/S or G_2/M) or cell differentiation, both of which are affected by p21 levels. It is attractive to speculate that the co-involvement of Ref-1 in BER and transcriptional control of p53 may represent a tightly regulated mechanism that provokes cell cycle arrest to allow DNA repair by the BER system. The existence of such a mechanism is still hypothetical.

Ref-1 has been implicated in the clonogenic survival or the clastogenic effect after treatment with certain DNA-damaging agents (Ono *et al.*, 1994; Walker *et al.*, 1994; Grosch *et al.*, 1998). In at least one case (Walker *et al.*, 1994), experiments were performed in HeLa cells, which are effectively p53 null, suggesting that effects of Ref-1 exclusive of p53 are involved. In such cells, Ref-1 down-regulation increased sensitivity to DNA-damaging drugs. In contrast, in our experiments apoptosis induced by p53 over-expression is decreased upon Ref-1 downregulation in H1299 cells. This discrepancy may be explained by the fact that in our case apoptosis is dependent upon p53 expression. However, DNA-damaging drugs can act upstream of p53 and may also induce p53-independent pathways that may be modulated by Ref-1. Additionally, Ref-1 has an endonuclease activity required for BER. Downregulation of Ref-1 in cells treated with DNA-damaging drugs is likely to reduce the ability of these cells to repair DNA, which can potentially lead to apoptosis.

How does Ref-1 modulate the activity of p53?

We originally identified Ref-1 through a biochemical screen for factors that can stimulate DNA binding by p53. Ref-1 was found to enhance DNA binding by wild-type p53 but not a C-terminally deleted form of p53 (p53Δ30). Similarly, Ref-1 was observed to stimulate transactivation of a reporter gene by wild-type p53, but not by p53Δ30 (Jayaraman *et al.*, 1997). This suggests that the *in vivo* effects of Ref-1 on p53 are the result of its stimulation of p53 binding to response elements within promoters, although more information is needed to confirm this possibility. In this study we further extend our knowledge about the mechanism that may allow Ref-1 to regulate p53 function by showing that Ref-1 and p53 interact *in vitro* and can be co-immunoprecipitated from extracts. The proportion of Ref-1 or p53 associated *in vivo* is low ($<$5%), although we cannot rule out the fact that we may not have found the optimal experimental conditions to identify a more extensive interaction between the two proteins. However, in contrast to other activators of p53, Ref-1 stimulates p53 at sub-stoichiometric ratios, which are in the catalytic range (Jayaraman *et al.*, 1997). Thus, very small amounts of Ref-1 may be sufficient to activate p53 *in vivo*. One possible way in which Ref-1 modifies the activity of its targets (c-*fos*, c-*jun*) is by altering their redox state (Xanthoudakis and Curran, 1992), which implies an enzymatic reaction and an unstable protein interaction. Interaction of Ref-1 with c-*fos* was only observed after chemical or UV protein crosslinking (Xanthoudakis and Curran, 1992). It is well established that the DNA-binding activity of p53 is strongly dependent on its redox state and that part of the stimulatory effect of Ref-1 involves a redox-dependent mechanism (Jayaraman *et al.*, 1997). Another possibility would be a modulation of p53 protein stability by Ref-1. Indeed, in Figure 2B, over-expression of Ref-1 slightly increases p53 protein levels. However, we observed that when Ref-1 expression

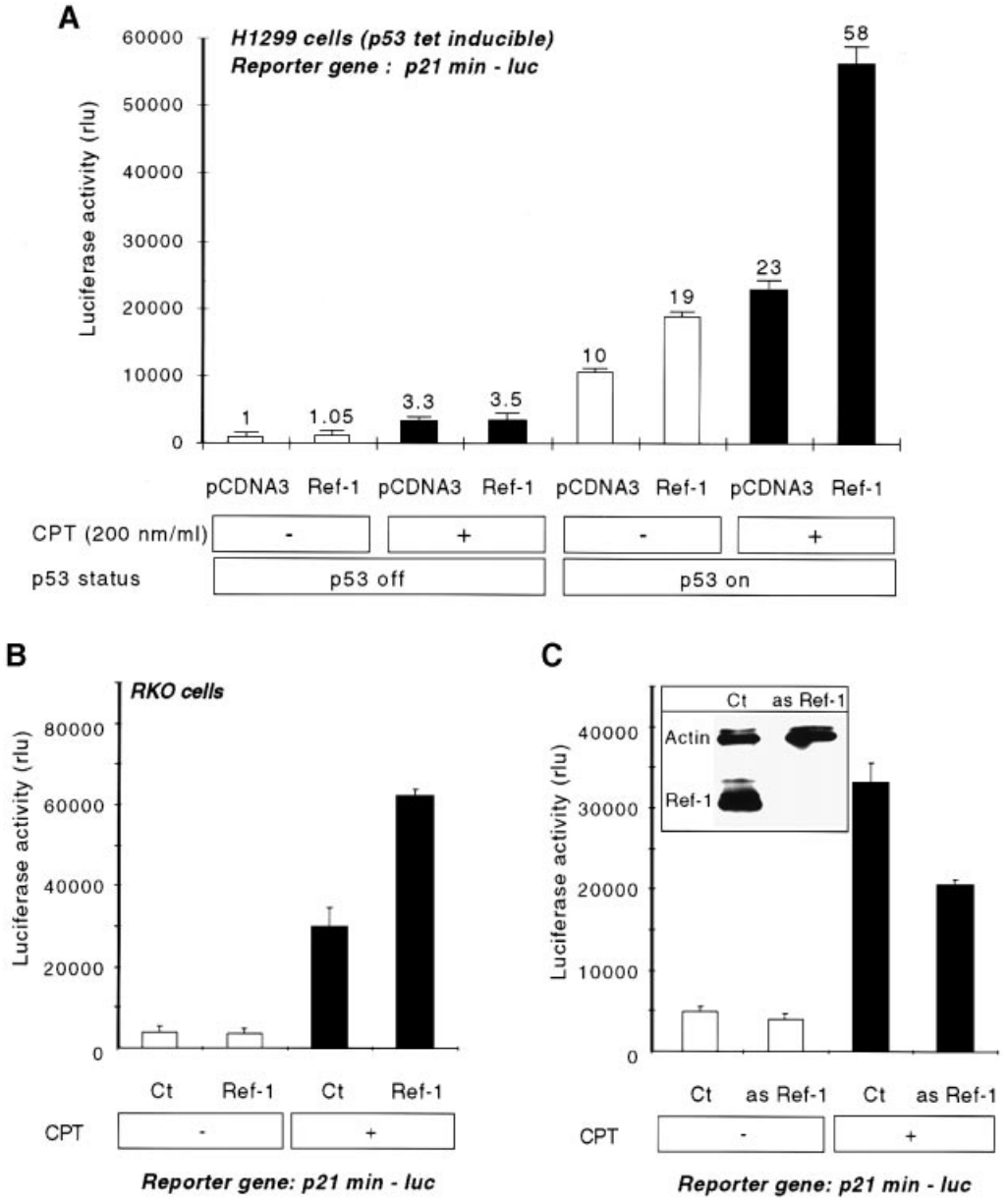


Fig. 8. Ref-1 cooperates with DNA-damaging drugs to activate p53-driven transcription. (**A**) H24-14 cells were transfected with a CMV-Ref-1 vector and the p21–luc reporter. After transfection (72 h), cells were treated (+) or not (–) with CPT (200 μM) and tetracycline was removed (p53 on) or not (p53 off). Cells were harvested 24 h later. Diagrams represent means in relative luminescence units (r.l.u.) and bars are standard deviations. Each diagram shows a representative experiment out of two, each done in triplicate. Numbers above the error bars indicate fold induction when compared with cells transfected with pCDNA3 in the absence of CPT and in the presence of tetracycline. (**B**) RKO cells were co-transfected with the p21 min–luc reporter gene and either with 1.5 μg of the pHook Ref-1 vector [Ref-1, (B)] or the pHook antisense Ref-1 vector [as Ref-1, (**C**)]. After transfection (24 h), cells were treated (+) or not (–) with CPT for 24 h. Inset in (C) shows Ref-1 and actin protein detected by Western blotting of protein extracts from RKO cells transfected by pCDNA3 (Ct) or antisense Ref-1 IRES GFP (as Ref-1). Diagrams represent means in r.l.u. and bars are standard deviations. Each diagram shows a representative experiment out of three, each done in triplicate.

was downregulated by antisense, while p53 transcriptional activity was markedly inhibited, there was no significant effect on p53 protein levels (Figure 6). Thus Ref-1 does not act via modulation of p53 stability in the cell lines that we used in our experiments. It remains possible, however, that in other cell types Ref-1 might affect p53 protein levels as well as activity. Together, these observations support the idea that a direct interaction between Ref-1 and p53, while possibly transient, is involved in its ability to stimulate p53 *in vivo*.

Is Ref-1 a component of the signal transduction pathway that activates p53 after DNA damage?

To link the effects of Ref-1 on p53 to the normal role of p53 as a stress response factor, we asked whether Ref-1 is involved in the activation of p53 following DNA damage. It was observed that in an inducible p53 cell line, CPT cooperates with Ref-1 to increase the transcriptional activity of p53. Furthermore, in RKO cells that contain an endogenous wild-type p53 protein which can be induced by DNA damage, Ref-1 also cooperated with CPT to

induce p53-dependent transcription. Perhaps more compelling was our observation that reduction of Ref-1 by antisense decreased transactivation by p53 in the presence of CPT. In these experiments, p53-dependent transcription was assessed with a reporter gene (p21 min–luc) containing only the p53-responsive element from the p21 promoter, thus reducing the likelihood that interference with other DNA sequences from the p21 promoter may mediate a p53-independent effect of either Ref-1 and CPT or both.

It is not yet obvious how cooperation between p53, Ref-1 and CPT might occur, nor whether other agents might be able to facilitate the effects of Ref-1 on p53. Our preliminary results suggest that oxidative stress leads to increased complex formation between p53 and Ref-1. Experiments are in progress to elucidate these observations further and to evaluate their physiological relevance. It was reported that the ability of Ref-1 to induce the AP-1 complex activity is further stimulated by TPA (Hirota *et al.*, 1997). Ref-1 function in that case is apparently regulated or restored by TPA via the activation of the thioredoxin reductase/thioredoxin system. Interestingly, the thioredoxin reductase gene is essential in yeast for the transcriptional activity of p53 (Casso and Beach, 1996). Experiments examining a possible role for a signal transduction pathway involving the thioredoxin reductase/thioredoxin system and Ref-1 are currently under way.

Materials and methods

Cell culture

RKO and H1299 cells were obtained from American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in the presence of 5% CO_2/95% air at 37°C. HA Ref-1 tetracycline-inducible cell lines were established as described before (Chen *et al.*, 1996). H24-14 (Chen *et al.*, 1996) and HA Ref-1 stable cell lines were cultured in the presence of puromycin (2 μg/ml), neomycin (500 μg/ml) and tetracycline (5 μg/ml).

Transfection and selection of transfected cells for RT–PCR or Western blot analysis

Transfections were carried out with a lipopolyamine-based protocol (Transfectam™) as described previously (Gaiddon *et al.*, 1994). For reporter gene assays, cells were transfected with 400 ng of reporter gene and 400 ng of expression vector. Once transfected, cells were isolated for further analysis, transfections were performed in 10-cm plates with antisense Ref-1 pHook vector or HA Ref-1 pHook vector (10–25 μg). After 12 h, cells were washed once with phosphate-buffered saline (PBS) and incubated for 42 h in fresh medium. For further analysis, transfected cells were isolated using a Capture-Tech pHook2 system (Invitrogene™). Briefly, cells were carefully harvested into 3 ml of PBS/3 mM EDTA, centrifuged for 5 min at 800 r.p.m. and washed once with the PBS/3 mM EDTA buffer. Cell pellets were resuspended in 1 ml of medium including serum and then 25 μl of magnetic beads were added. Cells were slowly rocked for 45 min at 37°C and pelleted for 1 min using a magnetic strand. Pellets were resuspended into fresh medium and then cells were pelleted again in the same way. This washing step was repeated 2 to 8 times, depending on the cell type and the transfection efficiency. After the last wash, cell pellets were evaluated visually and directly lysed into mRNA extraction buffer (200 μl) (Chomczynski and Sacchi, 1987) or protein sample buffer (50 μl).

mRNA extraction and RT–PCR analysis

RNA was extracted as described previously (Chomczynski and Sacchi, 1987). Two micrograms of total RNA were used for reverse transcription reactions with 100 U of *Mulerian myeloblastosis* virus reverse transcriptase (Promega) in a 30 μl reaction mix containing 20 U of RNAsin (Promega), 1 mM dithiothreitol (DTT), 0.5 mM each dNTPs, 8 μM random hexamers and 1× MuMLV buffer (Promega). Reaction mixtures were incubated at 42°C for 50 min and terminated by adding 70 μl H_2O and boiling for 5 min. PCRs were performed with 3 μl of the reverse transcription products and 1 U of AmpliTaq DNA polymerase (Perkin Elmer) in a 50 μl reaction mix (including 100 μM each dNTPs, 1.5 mM Mg^{2+}, 1× AmpliTaq DNA polymerase buffer and 15 pmol of each specific primer). Typical cycle parameters were 1 min at 94°C, 1 min at 55°C and 2 min at 72°C for 15 to 35 cycles followed by a final incubation at 72°C for 10 min. Ten microliters of each reaction mixture were electrophoresed through a 2% agarose gel, and the gel was photographed and subjected to densitometric analysis. Control experiments were performed to determine the range of PCR cycles over which the amplification efficiency remains constant and proportional to the amount of input RNA. Oligonucleotides used for p21, cyclophilin and cyclin G mRNA amplifications were respectively: 5′-GTGGACAG-TGAGCAGTTGAG-3′/5′-GCTGGTCTGCCGCCGTTTTC-3′; 5′-GG-GGAGAAAGGATTTGGCTA-3′/5′-ACATGCTTGCCATCCAGCCA-3′ and 5′-ATGACTGCAAGACTAAGGGA-3′/5′-CTTGCCAGAAGGTC-AGATCT-3′.

Far-Western analysis

Ref-1 and p53 proteins were purified as in Jayaraman *et al.* (1997), and wild-type and dl166 Mdm2 proteins were purified as in Shieh *et al.* (1997). Far-Western analysis was essentially as described (Jayaraman *et al.*, 1998). Proteins were subjected to SDS–PAGE, followed by transfer to nitrocellulose. After staining with Ponceau-S to identify the polypeptides, the filter was incubated in denaturation buffer (6 M guanidine–HCL in PBS) twice for 5 min at 4°C. Filters were then incubated in serial dilutions of denaturation buffer in PBS with 1 mM DTT. After blocking the membrane in PBS containing 0.5% Tween 20 and 5% non-fat dry milk (NFDM) for 45 min at 20°C the blot was washed twice with PBS/Tween 20/0.25% NFDM, and then incubated with p53 (0.2 μg/ml) in PBS containing 0.5% Tween 20, 0.25% NFDM, 1 mM DTT and 0.5% phenylmethylsulfonyl fluoride (PMSF) for 2 h. The filter was washed 4× in PBS containing 0.5% Tween 20 and 0.25% NFDM, before being probed with PAb 421 as primary antibody and goat anti-mouse as secondary antibody. Proteins were detected using ECL (Amersham, IL).

Immunoprecipitation and immunoblotting

Cells were lysed in 300 μl of lysis buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.5 mM PMSF, and protease inhibitor) and the extracts were centrifuged at 8000 *g* for 15 min to remove cell debris. Protein concentrations were determined using the Bio-Rad assay (Bio-Rad, CA). Immunoprecipitations of p53 proteins were performed by incubating 1.5 mg of whole-cell extract with 100 ng of each of the following purified anti-p53 mAbs: mAb240, mAb421 and mAb1801, and rocking at 4°C for 1 h. Then, 20 μl of protein A–Sepharose beads (Pharmacia, Uppsala, Sweden) were added and the samples were rocked at 4°C for 1 h. The samples were washed 4× with 1 ml of wash buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, 10% glycerol, 150 mM NaCl), the excess liquid was aspirated, and 30 μl of 2× sample buffer (DiComo *et al.*, 1999) were added. Samples were heated to 95°C for 5 min, centrifuged for 3 min at 13 000 *g*, and electrophoresed through a 10% SDS–polyacrylamide gel. Protein gels were transferred to nitrocellulose membranes (Schleicher & Schuell, NH). For p53 detection, a mixture of p53 mAb-containing supernatants (pAb421, pAb1801 and pAb240) was used, each at a 1:4 dilution. For Ref-1 detection, a pAb was used at 1:2000 dilution; for p21 detection, the Ab1 pAb (SantaCruz, CA; 1 mg/ml) was used at 1:200, and for the detection of actin a pAb (Sigma, MO) was used at a dilution of 1:200. For detection of p53 in Ref-1 immunoprecipitates, we used a secondary anti-mouse antibody that specifically recognizes the mouse κ light chain (1/1000, Pharmingen, CA). Proteins were visualized with an enhanced chemiluminescence detection system (Amersham, IL).

Expression vectors and reporter genes

The p53 expression vector (pCMVp53wt) and p21–luc, GADD45–luc and Bax–luc have been described previously (Friedlander *et al.*, 1996). p21–luc, Bax–luc and GADD45–luc reporters contain, respectively, the p21 promoter (2400 bp), the Bax promoter (bp –687 to –318) or fragment of GADD45 gene spanning the intronic p53-responsive element. The p21 min–luc reporter gene was prepared from oligonucleotides containing the p53-responsive sequence 5′-GAACATGTCCCAACATGTTG-3′ from the p21 promoter. This sequence was inserted into a pGL3 reporter construct containing the minimal c-*fos* promoter (sequence from bp –53 to +42) upstream of luciferase cDNA. CMV–luc contains the luciferase cDNA driven by the cytomegalovirus early promoter. A tetracycline-

inducible HA Ref-1 expression vector was prepared from an HA Ref-1 PCR fragment with *Eco*RI and *Xba*I ends, inserted into the *Eco*RI–*Xba*I sites of the p10-3 vector described previously (Gossen and Bujard, 1992). Sense and antisense HA Ref-1 pHook-expression vectors were prepared from the PCR fragment subcloned into the *Sma*I site of the pHook2 vector (Invitrogene, CA). Sense and antisense Ref-1 GFP vectors were prepared by insertion into an *Eco*RV site of the pIRES–GFP vector (Clontech, CA).

Apoptosis assays

H1299 cells in 10-cm plates were co-transfected with 7 μg of pCMVp53wt, and a construct (7 μg) containing either the Ref-1 cDNA or the antisense Ref-1 pIRES–GFP vectors (Clontech, CA). Where appropriate, 7 μg of GFP-containing IRES construct without Ref-1 sequences was included. Cells were observed under epifluorescence (Nikon, DIAPHOT 300) 72 h post-transfection and images photographed with an Optronics 3CCD video camera linked to a Macintosh computer. Under each experimental condition three plates were utilized and 500 GFP-stained cells were counted in randomly selected fields from each plate. Among the GFP-stained populations, apoptotic cells were identified by the presence of apoptotic bodies or membrane blebbing.

Cells were also subjected to FACS analysis as described by Lamm *et al*. (1997). Briefly, 72 h after transfection, cells were fixed in 2% paraformaldehyde in a fixative buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES pH 6.8) for 20 min and then in 95% methanol for 1 h. Fixed cells were washed 3× with PBS and exposed to PI (60 μg/ml) and RNase A (50 μg/ml) for 30 min before counting by FACS (FACScalibure, Becton Dickinson). Cell harvesting and FACS analysis were performed the same day to avoid loss of GFP staining. Cells (100 000) were gated for GFP staining with a 530/20 nm bandpass filter and then analyzed for DNA content (PI) with a 610 nm longpass filter. Excitation wavelength of 488 nm was used for GFP and PI. Data were analyzed using CELLQuest software (Becton Dickinson). From the DNA content profile, the sub-G_1 population of cells was gated and quantified.

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