Identification of redox/repair protein Ref-1 as a potent activator of p53

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p53 can be isolated from cells in a form that is inert for binding to DNA but that can be stimulated dramatically by phosphorylation, antibody binding, or short single strands of DNA. This suggests that upon genotoxic stress, cells can convert latent p53 to one that is active for DNA binding. Surprisingly, we observed that latent p53 is as effective in activating transcription *in vitro* as is active p53. We found that HeLa nuclear extracts can stimulate DNA binding by latent p53 and have purified from them a p53-stimulating protein that we have determined to be the product of the *Ref-1* gene. Interestingly, Ref-1 is a dual function protein that can both regulate the redox state of a number of proteins and function as a DNA repair (A/P) endonuclease. We observed that oxidized forms of full-length and carboxy-terminally truncated p53 (p53Δ30), which are inactive for DNA binding, are both stimulated by the Ref-1 protein. However, in the presence of reducing agent, Ref-1 is an extremely potent stimulator of full-length p53 but not p53Δ30. These and additional data indicate that Ref-1 protein stimulates p53 by both redox-dependent and -independent means and imply a key role for it in p53 regulation. Importantly, we have also determined that Ref-1 can stimulate p53 transactivation *in vivo*. This is the first example of a noncovalent protein modifier of p53 function identified in cells.

[Key Words: Ref-1 protein; redox/repair; p53 activation; redox dependent; independent stimulation]

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The p53 gene product plays a complex and important role in cells. In response to stress signals such as DNA damage or hypoxia, p53 acts as a brake by inducing either cell cycle arrest or apoptosis, thereby serving to maintain genetic stability [for review, see Gottlieb and Oren 1996; Ko and Prives 1996]. p53 has the features and functions of a sequence-specific transcriptional activator (Vogelstein and Kinzler 1992). Several genes have been identified that contain p53 response elements that conform to its rather loose consensus binding site. These genes, which are activated by p53 in response to DNA damage, include *p21/Waf1/Cip1*, *mdm2*, *Gadd45*, *cyclin G*, *Bax*, *IGFBp3*, and others (Ko and Prives 1996 and references therein).

The carboxyl terminus of p53 has been shown to play an important role in controlling p53 DNA-binding function. Modification of a highly basic region within the carboxy-terminal 30 amino acids of the p53 polypeptide can convert p53 from an inert to an active state for DNA binding. Phosphorylation of (Hupp et al. 1992; Delphin and Baudier 1994) antibody binding to (Hupp et al. 1992; Halazonetis et al. 1993) or deletion of (Hupp et al. 1992; Halazonetis and Kandil 1993; this study) this region can

strongly stimulate p53 DNA binding *in vitro*. Additionally, DNA binding by p53 has been shown to be stimulated by a protein fragment spanning the entire carboxyl terminus (311–393) (Jayaraman and Prives 1995) or smaller peptides spanning a region within the carboxy-terminal 30 amino acids (Hupp et al. 1995; Shaw et al. 1996; this study). Another effector of p53 DNA binding that operates through the p53 carboxyl terminus has been shown to be short single strands (Jayaraman and Prives 1995). Furthermore, phosphorylation of a single cyclin-dependent kinase (CDK) site at Ser-315 that lies between the DNA-binding domain and the tetramerization domain can both stimulate and alter p53 DNA binding (Wang and Prives 1995). Thus, although the central DNA-binding domain alone can bind well and sequence specifically to DNA, this domain is subjected to regulation by the carboxyl terminus when it is in the context of the full-length protein. Communication between different regions of p53 serves to regulate and fine-tune binding by the full-length protein. These observations, which have led to a model for allosteric regulation of p53 (Halazonetis and Kandil 1993; Hupp and Lane 1994), imply that there may be agents in cells that, upon genotoxic insult, are capable of effecting the switch between latent and active forms.

Given the potency of p53, it is not surprising that the

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redox state of the cell is superimposed upon manipulation of its conformation by varied cellular effectors. Treatment of p53 with oxidizing agents or metal chelators renders the protein incapable of DNA binding, whereas reduction enhances it (Hainault and Milner 1993; Delphin et al. 1994). Additionally, mutation of critical cysteine residues in the murine p53 DNA-binding domain abolishes or alters DNA binding by the protein (Rainwater et al. 1995). Free oxygen radicals are produced during many stress conditions, including hypoxia and ionizing radiation. p53 activity may be regulated by both the presence of oxygen intermediates and the counterbalancing cellular reducing response. Because p53 is regulated by its state of redox it is interesting to consider the possibility that one or more factors exist in cells that can modulate a conversion from a more oxidized to a more reduced state. This is a particularly attractive idea, as it was shown recently that hypoxia induces the ability of wild-type p53 to cause apoptosis in human tumors (Graeber et al. 1996).

As part of an effort to determine whether regulation of p53 DNA binding can affect its function as a transcriptional activator in vitro we have discovered that a factor, Ref-1, identified previously as a redox/repair protein (Xanthoudakis et al. 1994 and references therein), is an extremely potent activator of latent p53 protein such that activation is achieved by substoichiometric amounts. Our data showing that Ref-1 stimulates p53 both by redox-dependent and -independent means in vivo and in vitro suggest that this factor may be a critical sensor of genotoxic stress that can activate latent p53 in cells.

Results

Active and latent forms of p53 differ in DNA-binding ability but activate transcription in vitro to similar extents

Our original goal was to characterize the abilities of latent and active forms of p53 to activate transcription from a p53 responsive promoter in vitro. To purify these two forms of p53 we constructed a recombinant baculovirus expressing p53 containing an epitope tag (flu) at the amino terminus. Figure 1A shows a silver-stained gel with the levels of the two forms of p53 protein used in the experiments shown in Figures 1 and 2. The pAb421 epitope is within the carboxy-terminal 30 amino acids of p53, the autoinhibitory region that negatively regulates p53 sequence-specific DNA binding, and both pAb421 (Hupp et al. 1992; Halazonetis et al. 1993) and the pAb421 epitope peptide (Hupp et al. 1995; Shaw et al. 1996; this study) activate p53 DNA binding. As expected, we found dramatic differences in the relative binding affinities of the two forms of p53 for DNA containing the GADD45 consensus site by electrophoretic mobility shift assay (EMSA; Fig. 1B). p53 purified by using the pAb421 peptide bound DNA far more efficiently than did flu peptide-eluted p53. The striking difference in DNA-binding ability by the two forms of p53 was

observed with several other p53 binding site-containing oligonucleotides, including the p21/WAF1 site (data not shown).

When these two sources of p53 were compared for their ability to activate transcription in vitro, however, very similar levels of activated transcription were observed in both cases over the range of p53 used (Fig. 2A,B). We then tested whether a fragment of p53 that spans the carboxy-terminal domain (residues 311–393) or a shorter peptide (residues 372–382), both of which strongly stimulate DNA binding by latent p53 (Fig. 2C), were capable of stimulating transcriptional activation by latent flu-p53 in vitro. When either peptide was added to in vitro transcription mixtures, no further enhancement of transactivation by p53 was observed (Fig. 2D). Additionally, the monoclonal antibody pAb421, which stimulates p53 DNA binding, was unable to activate p53 further in in vitro transcription assays (C. Cain and C. Prives, unpubl.). These results suggest two alternate possibilities: Either HeLa extracts contain a factor(s) that is capable of activating latent p53, or DNA binding is not a rate-limiting factor in p53-transactivated transcription. Evidence supporting the first of these assumptions is shown below.

Purification and identification of p53-activating factor Ref-1 from HeLa nuclear extracts

We checked whether the phosphocellulose P-11 fractions used in the transcription assays were capable of activating p53 DNA binding by EMSA. As shown in Figure 3A, the phosphocellulose P11.5 fraction (0.5M KCl eluate) markedly stimulated DNA binding by p53 (cf. lane 1 to lanes 2 and 3). Competition assays using excess unlabeled oligonucleotides containing either wild-type or mutant p53 binding sites showed that the stimulated binding was sequence specific (data not shown). This observation led to the decision to purify and identify the p53 stimulatory activities in the HeLa P11.5 fraction.

A preliminary investigation of the properties of the p53 activating factor on a selection of columns was first carried out to derive the final purification scheme outlined in Figure 3B. In all chromatographic steps individual fractions were analyzed for stimulation of p53 DNA binding by EMSA, and active fractions were pooled, dialyzed, and loaded onto the next column. We were able to purify the stimulatory activity such that the final Superdex 75 fraction consisted of a single predominant polypeptide with an estimated relative molecular mass of 37 kD based on its electrophoretic mobility (Fig. 3C), and to show that the stimulatory activity coeluted with this protein (data not shown). The 37-kD polypeptide was isolated and subjected to proteolytic digestion. Automated protein sequencing of one of the resulting peptides yielded a 6-amino-acid sequence corresponding to a region (residues 80–85) within a known protein, Ref-1 (Xanthoudakis and Curran 1992). Three additional lines of evidence confirmed the stimulatory factor as Ref-1. First, the electrophoretic migration of the polypeptide that we purified was similar to that reported for

Figure 1. Differential abilities of active and latent immunopurified p53 to bind to DNA. (A). Flu-tagged p53 was purified from infected insect cells on either a pAb421 column or a mAb 12.CA5 column and increasing quantities (12.5, 25, 50, and 100 ng) were analyzed by SDS-PAGE. (B, top) pAb421 peptide-eluted (open box) or flu peptide-eluted (shaded diamond) p53 proteins (6.25, 12.5, 25, 50, and 100 ng) were bound to 32 P-labeled GADD45 oligonucleotides and analyzed by EMSA and autoradiography. (Bottom) Graphic representation of Phosphor-Imager-quantified units of DNA protein complexes shown at the top.

Ref-1 by Xanthoudakis and Curran (1992). Second, the major protein of the peak Superdex-75 fraction was rec-

ognized by a polyclonal anti-Ref-1 antibody (Fig. 3D, lanes 1,2). Third, recombinant Ref-1 purified from bacteria stimulates p53 DNA binding in a manner that is similar to that of the Superdex-75 fraction (see below).

The purification of Ref-1 from the P11.5 fraction of HeLa nuclear extracts suggested that this was the key stimulatory factor that activated latent p53 in this crude fraction. Immunodepletion of this fraction with affinity-purified anti-Ref-1 antibody showed that there was a marked reduction in the p53 stimulatory activity in extracts treated with the Ref-1 antibody when compared to a similar amount of extract that was treated with an unrelated antibody (data not shown).

Ref-1 has been identified previously as having two different functions. Xanthoudakis and Curran (1992) purified Ref-1 from HeLa cell extracts as a factor that increases the DNA-binding activity of AP-1. They showed further that Ref-1 stimulates AP-1 by providing a redox activity leading to the transfer of hydrogen to a conserved cysteine residue within the DNA-binding domain of AP-1 (Abate et al. 1990; Xanthoudakis et al. 1994). Interestingly, however, a number of laboratories identified this gene product through another seemingly completely independent activity, namely its function as a DNA repair protein classified as a class II hydrolytic apurinic/apyrimidinic (A/P) endonuclease; hence, its alternative names APE (Dempfle et al. 1991), HAP-1 (Robson and Hickson 1991), or APEX (Seki et al. 1991). Ref-1 is thus a dual function protein with both redox and DNA repair activities. As shown below, we believe that Ref-1 may possess a third function, namely the ability to stimulate p53 in a redox-independent manner.

Recombinant Ref-1 activates p53 DNA binding

The identity of the p53 stimulating factor was finally verified when we tested the effect of bacterially produced and purified His-tagged Ref-1 on p53 by EMSA. As seen in Figure 4A (cf. lanes 2 with 3 and 4) recombinant Ref-1 was capable of significantly stimulating DNA binding by full-length p53. The magnitude of stimulation ranged between ~10- and 30-fold. Ref-1 also strongly stimulated p53 binding to other sites, that is, *p21*, *bax*, and ribosomal gene cluster (*RGC*) (not shown). However, Ref-1 was incapable of stimulating binding of a carboxy-terminally deleted p53 (p53 Δ 30), which lacks the carboxy-terminal 30 amino acids (Fig. 4A, cf. lane 5 with lanes 6 and 7). As is evident from Figure 4A, interaction between p53 and Ref-1 is likely to be of a transient nature because there is no change in mobility between the unstimulated and stimulated p53-DNA complexes. The minor rapidly migrating DNA-protein complex seen in this figure (indicated by the arrow) and in Figure 5, A and B, is attributable to the Ref-1 protein itself binding nonspecifically to the labeled probe (data not shown). Although two p53-specific monoclonal antibodies (pAb1801 and pAb421) were capable of supershifting the Ref-1-stimulated complex, similar addition of anti-Ref-1-specific antibody to the reaction had no effect, indicating that Ref-1 was not part of the activated p53-DNA complex (data not

Figure 2. Similar transcriptional activation by active and latent forms of p53. (A) Increasing amounts (5, 6.25, 12.5, 25, 50, and 100 ng) of either pAb421 peptide (lanes 3–8) or flu peptide-purified (lanes 9–14) p53 proteins, as indicated, were incubated in reaction mixtures containing pWAF–MLT (test p53 responsive template, thin arrow) and pML cassette (reference template, thick arrow), buffers, [α^{32} P]CTP, and HeLa nuclear fractions as described in Materials and Methods. Reaction products were analyzed on 4% urea–acrylamide gels. Lanes labeled basal contained mixtures with no p53. (B) The extent of activation of both forms of p53 over basal levels were quantitated by PhosphorImaging the transcripts shown in A and graphed after normalizing to the reference template. (Solid square) pAb421 peptide-purified protein; (shaded circle) flu peptide-purified protein. (C) A small basic peptide or a fragment from the p53 carboxyl terminus (311–393) stimulated latent p53 DNA binding. Increasing amounts (500 ng, 1 μ g, and 2 μ g) of an 11-mer peptide that spans the pAb421 epitope on p53 (KKGQSTSRHKK) or of a p53 fragment (311–393) (200, 400, and 800 ng) were added to reaction mixtures containing 25 ng of p53 and a 32 P-labeled GADD45 oligonucleotide followed by EMSA analysis as in Fig. 1B. (D) Similar quantities of small peptide or carboxy-terminal fragment were added to in vitro transcription mixtures and analyzed as in A. Thin and thick arrows indicate test and reference transcripts, respectively, as in A.

shown). Furthermore, we were not able to detect a stable interaction between p53 and Ref-1 either in vitro or in vivo (data not shown). Ref-1 stimulatory activity was inactivated by heat treatment (95°C for 5 min), although it was capable of activating p53 at 37°C, albeit to a slightly lesser extent than at 25°C (data not shown).

When we titrated the p53 stimulatory activity of Ref-1 we were surprised to find that as little as 55 pg of Ref-1 resulted in increased binding by 25 ng of p53. Half-maximal stimulation of that amount of p53 was achieved by 150–200 pg of Ref-1 (Fig. 4B), that is, at a molar ratio of

Ref-1 to p53 of ~1:200. It is acknowledged, however, that we cannot evaluate easily the fraction of p53 molecules that is competent for DNA binding. Nevertheless, stoichiometric quantities of either short single strands of DNA or the p53 carboxyl terminus (Jayaraman and Prives 1995) or a short basic peptide from a region within the carboxy-terminal 30 amino acids (Fig. 2C; Hupp et al. 1995) are required for stimulating p53 DNA binding.

Xanthoudakis et al. (1994) showed previously that the redox and A/P endonuclease activities of the 318-amino-acid Ref-1 protein are specified by nonoverlapping do-

Figure 3. (See facing page for legend.)

Figure 4. Recombinant Ref-1 is a potent stimulator of p53 DNA binding. (A) DNA binding by 25 ng of full-length p53 or of 5 ng of p53 Δ 30 (1–363) in the absence or presence of 50 and 100 ng of Ref-1, as indicated, was analyzed as in Fig. 1B. Lane 1 contains probe alone. The arrow indicates nonspecific Ref-1-DNA complex. (B) DNA binding by 25 ng of p53 as in Fig. 1A was examined in the absence (lane 1) or the presence of the indicated quantities of either full-length Ref-1 or carboxy-terminally truncated Ref-1 (1–224).

mains that map to regions within the amino and carboxyl termini, respectively. A truncated Ref-1 lacking the carboxy-terminal 94 amino acids (Ref-1 Δ C) containing a disrupted A/P endonuclease domain was still capable of stimulating p53 (Fig. 4B). However, markedly greater quantities of Ref-1 Δ C were required for full stimulation of p53. Thus, although truncated Ref-1 stimulated p53 at stoichiometric levels, full-length Ref-1 exerted its effect in the catalytic range.

Redox-dependent and -independent stimulation of p53 by Ref-1

Because Ref-1 strongly stimulated p53 in the presence of a reducing agent such as dithiothreitol (DTT), we considered the possibility that its activation of p53 might be independent of its function as a redox protein. We therefore examined the effect of Ref-1 on DNA binding by oxidized p53 in the presence and absence of reducing agent. As carboxy-terminally deleted p53 (p53 Δ 30) can-

not be activated by Ref-1 in the presence of DTT, we also examined DNA binding by the oxidized form of this protein in the absence and presence of Ref-1.

Figure 5A shows the effect of Ref-1 or DTT, or the combined effect of both agents, on DNA binding by oxidized p53. Confirming previous reports (Hainault and Milner 1993), oxidized p53 bound very poorly to DNA (lane 2). In the presence of increasing amounts of Ref-1, binding was enhanced ~20-fold. The addition of increasing amounts of DTT also augmented DNA binding by oxidized p53. DTT stimulated binding to an even greater extent over that seen with Ref-1 such that maximal stimulation was on the order of 50-fold. Combining both DTT and Ref-1 led to a further stimulation of binding to a considerable extent over and above what was seen with either alone. Quantitation by PhosphorImaging indicated that binding in the presence of both factors was 8- to 10-fold greater than the summed enhancement of binding by either effector.

Carboxy-terminally deleted p53 (p53 Δ 30) has been

Figure 3. Purification of Ref-1 as the p53 activating protein from HeLa P11.5 fraction. (A) DNA-binding reaction mixtures as in Fig. 1B contained 1 and 2 μ l of the HeLa P11.5 fraction either with (lanes 2,3) or without (lanes 4,5) 25 ng of p53. Lane 1 contains reaction mixture with p53 alone. Reactions were analyzed by EMSA and autoradiography as in Fig. 1A. (B) Protocol for purification of p53 DNA-binding stimulatory activity from HeLa nuclear extract. Fractions from each step were assayed for stimulation of p53 DNA binding as in A. Peak stimulatory fractions were pooled, dialyzed against BC100 buffer, and applied to the next column. (C) Aliquots of peak fraction from each step in B were analyzed by SDS-PAGE and silver staining. (D) Recombinant His-tagged Ref-1 or Superdex-75 peak fraction were subjected to SDS-PAGE, transferred to nitrocellulose, and the resulting blots were probed with either anti-Ref-1 antibody or nonspecific control antibody.

Figure 5. Ref-1 and DTT cooperatively stimulate DNA binding by oxidized p53. (A) p53 protein (25 ng) prepared in the absence of reducing agent was bound to DNA in the absence (lane 2) or presence of increasing amounts of Ref-1 (50, 100, and 200 ng; lanes 3–5), 2, 5, and 10 mM DTT (lanes 6–8), or the same range of Ref-1 in the presence of 2 (lanes 9–11), 5 (lanes 12–14), and 10 mM (lanes 15–17) DTT as in Fig. 1B. Lane 1 contains probe alone. (B) p53Δ30 (20 ng) was prepared in the absence of reducing agent and bound to DNA either alone or in the presence of Ref-1 and/or DTT as in A.

shown to be constitutively active for binding. Further increase of its binding ability by either Ref-1 (see Fig. 4A) or the p53 carboxy-terminal peptide (p53 residues 311–393) (L. Jayaraman and C. Prives, unpubl.) is not possible. These experiments, however, were all performed using reduced p53Δ30. Figure 5B shows binding by oxidized p53Δ30 and the relative effects of Ref-1 and DTT on this binding. Oxidized p53Δ30 showed very little binding to DNA (lane 2), and titrating increasing quantities of Ref-1 weakly stimulated binding by p53Δ30. However, the addition of DTT dramatically increased the ability of p53Δ30 to bind to DNA. In contrast to what we observed with full-length p53, addition of Ref-1 in the presence of DTT did not significantly enhance binding of p53Δ30 above what was seen with DTT alone, consistent with our earlier observation that p53Δ30 cannot be stimulated by Ref-1 under normal DNA-binding conditions in reducing buffers.

The postulated nonredox function of Ref-1 is perhaps akin to the ability of carboxy-terminal peptides or antibodies to stimulate latent p53. When we compared the effect of two known activators of p53, pAb421, and the carboxy-terminal peptide, on oxidized p53, in the presence or absence of Ref-1 (Fig. 6A) and DTT (Fig. 6B) we found that neither agent could activate oxidized p53 (Fig. 6; A, lanes 3, 4, 8, 9, and B, lanes 4, 5, 10, 11). In contrast, as shown above, Ref-1 stimulated DNA binding by oxi-

dized p53 (Fig. 6A, lane 5). The addition of pAb421 or p53 carboxy-terminal peptide in the presence of Ref-1 had no further stimulatory effect (Fig. 6A, cf. lane 5 with lanes 6 and 7 and 10 and 11). However, although the addition of DTT stimulated p53 (Fig. 6B, lanes 2,3), titrating into the reaction either pAb421 or p53 carboxy-terminal peptide, resulted in a dramatic further enhancement of binding (cf. lanes 2 and 3 with lanes 6–9 and 12–15). Thus, Ref-1 by itself was able to both reduce p53 and decrease the carboxy-terminally derived inhibition of p53, such that its binding could not be augmented further by either carboxy-terminal peptide or pAb421. DTT can only reduce p53 (albeit more efficiently than can Ref-1) and make it more accessible for manipulation by other effectors. Taken together, our data provide strong support for the likelihood that Ref-1 can stimulate reduced p53 in a manner akin to other activators that work through the p53 carboxyl terminus. Our results therefore suggest that Ref-1 can stimulate p53 both by a redox-dependent mechanism and by a novel redox-independent activity.

Ref-1 can stimulate wild-type but not mutant p53 in vivo

Our results showing that Ref-1 is a critical factor that can stimulate p53 binding and activation in vitro prompted us to determine whether a relationship be-

tween the two proteins might be identified *in vivo*. To this end we conducted transient transfection experiments testing the effect of Ref-1 on the ability of p53 to activate transcription from promoters containing p53 responsive elements. p53-null Saos-2 and H1299 cells were transfected with plasmids expressing p53 and Ref-1, either separately or together, along with a luciferase reporter construct driven by a segment of the cyclin G promoter (Fig. 7). This reporter construct was used be-

cause (1) it has been shown to be an endogenous target of p53 (Okamoto and Beach 1994; Zauberman et al. 1995), and (2) it contains a significant extent of the cyclin G promoter. Thus, it can be viewed as a physiologically relevant example of the corresponding p53 cellular target. Upon cotransfection of Ref-1 and p53 it was reproducibly observed that in both H1299 cells (Fig. 7A) and Saos-2 cells (Fig. 7B) there was significantly better activation of the cyclin G promoter than when p53 was introduced alone. As was expected, a mutant p53_{his175} neither activated this promoter nor was stimulated by Ref-1 in transfected H1299 cells (Fig. 7C). The cyclin G promoter was itself not significantly affected by expression of Ref-1 over a wide range of Ref-1 tested (data not shown), lending credence to the likelihood that Ref-1 was exerting its stimulatory effect directly on p53. Additionally, transfection experiments with the p21 promoter indicate that Ref-1 can cooperate with p53 in activating this promoter in both H1299 and Saos2 cells (data not shown). Importantly, consistent with the *in vitro* results obtained, Ref-1 did not stimulate the carboxy-terminally truncated form of p53, p53 Δ 30 (Fig. 7D), at all concentrations tested. This result lends further support to the likelihood that Ref-1 can stimulate p53 in a redox-independent manner (see Discussion). Our transfection experiments therefore show that Ref-1 and p53 can interact functionally in cells and therefore support the possibility that there is a role for Ref-1 in p53 function *in vivo*.

Discussion

The demonstration that Ref-1 is a noncovalent activator of p53 has important implications. It has been observed that phosphorylation of sites within the carboxyl terminus of p53, or deletion of the carboxy-terminal 30 amino acids of p53, leads to increased specific DNA binding *in vitro*. However, p53 constructs introduced into cells, either with mutated carboxy-terminal phosphorylation sites (Slingerland et al. 1993; Fiscella et al. 1994; Marston et al. 1994; L. Ko and C. Prives, unpubl.), or deleted carboxy-terminal 30 amino acids (L. Ko, X. Chen, and C. Prives, unpubl.), show similar transactivation abilities when compared to wild-type p53. Although it remains possible that efficiency of DNA binding is not the limiting factor in p53 activation function *in vivo*, the identification of Ref-1 as a novel stimulator of wild-type but not, for example, p53 Δ 30, might provide an explanation for the lack of concordance between experiments measuring DNA binding *in vitro* and transactivation *in vivo*.

Redox-dependent regulation of p53 by Ref-1

p53 belongs to a class of transcriptional regulatory proteins that are subject to redox modulation. We found that oxidized p53 was severely defective for DNA binding and could not be stimulated by either pAb421 or the carboxy-terminal peptide. This would suggest that an ordered series of alterations or regulatory events occur in cells when p53 is induced. Reduction of p53 would be

Figure 6. Evidence for redox-independent stimulation of p53 DNA binding by Ref-1. (A) Oxidized p53 (25 ng) was bound to DNA in the absence (lane 2) or presence of either 2 or 4 μ l of pAb421 (lanes 3,4) or 100 or 300 ng of p53 carboxy-terminal fragment (311–393) (lanes 8,9) or Ref-1 (lane 5). Lanes 6 and 7 contain p53–DNA complexes in the presence of Ref-1 with 2 or 4 μ l of pAb421. Lanes 10 and 11 contain p53–DNA complexes with Ref-1 in the presence of 100 or 300 ng of p53 carboxy-terminal fragment (311–393). Lane 1 contains probe alone. (B) Oxidized p53 (25 ng) was bound to DNA in the absence (lane 1) or presence of 5 or 10 mM DTT (lanes 2,3) or 100 or 300 ng of p53 carboxy-terminal fragment (311–393) (lanes 4,5) or 2 or 4 μ l of pAb421 (lanes 10,11). Lanes 6, 7, 12, and 13 contain p53–DNA complexes in the presence of 5 mM DTT with either 100 or 300 ng of p53 carboxy-terminal fragment (311–393) or 2 or 4 μ l of pAb421, as indicated. Lanes 8, 9, 14, and 15 contain p53–DNA complexes in the presence of 10 mM DTT and similar amounts of p53 carboxy-terminal fragment (311–393) or pAb421, as mentioned above.

Figure 7. Ref-1-mediated stimulation of transactivation by wild-type p53 but not p53 Δ 30 in transfected cells. (A–C) H1299 (A,C), or Saos2 (B) cells were transiently transfected with wild-type p53 (A,B; 3 μ g in Saos2 cells; 2.5 μ g in H1299 cells) or mutant p53_{his175} into H1299 cells (C; 2.5 μ g expression plasmid) or Ref-1 expression plasmid (2.5 μ g) or both p53 and Ref-1 expression plasmids. (Bar 1) Control vectors; (bar 2) p53 expression plasmid; (bar 3) Ref-1 expression plasmid; (bar 4) p53 and Ref-1 expression plasmids. (D) H1299 cells were transfected with either 2.5 μ g of wild-type p53 (+++) or 10 ng (+), 50 ng (++) or 2.5 μ g (+++) of p53 Δ 30-expressing plasmids. Ref-1 expression plasmid (2.5 μ g) was cotransfected as indicated. In all cases, total DNA transfected was normalized with equivalent amounts of control parental vectors. The reporter construct used was cyclin G-luc (1 μ g). Luciferase activity is represented as fold transactivation relative to samples transfected with reporter constructs and parental control plasmids. The fold activation represents an average of triplicate samples.

required prior to switching the conformation of the protein from one that is incapable of DNA binding to one that binds DNA efficiently. Redox regulation of p53 is probably mediated through one or more of the conserved cysteine residues in its DNA-binding domain (Rainwater et al. 1995). It is likely that alterations in the redox environment within cells could dictate to a significant extent the transactivation function of wild-type p53. Whether such changes do occur within normal circumstances within cells are for the present open to speculation. Given the fact that the concentrations of redox regulators such as glutathione (Bellomo et al. 1992) and Ref-1 (Xanthoudakis et al. 1992) are higher in the nucleus than in the cytoplasm, the very entry of nascent p53 into the nucleus would favor its reduction.

A new activity of Ref-1: redox-independent modulation of p53

Ref-1 was identified as a factor that stimulates AP-1 by reduction of a conserved cysteine residue within the DNA-binding domains of Fos and Jun (Xanthoudakis et

al. 1992). However, our data indicate that activation of p53 by Ref-1 has two separate components, one redox-dependent and the other redox-independent. Supporting this assumption is the fact that although substoichiometric quantities of Ref-1 are capable of stimulating p53, markedly greater amounts of Ref-1 are required for activation of AP-1 (Xanthoudakis et al. 1992). High levels of Ref-1 are necessary for its stimulation of oxidized p53, which presumably requires primarily the redox component (see Fig. 5A). Experimental evidence that supports a redox-independent p53 stimulatory function of Ref-1 includes the following: First, although both Ref-1 and DTT are capable of activating oxidized p53, the two together produce dramatically increased binding. It is not likely that DTT is simply regenerating the reducing ability of Ref-1: Other stimulators of DNA binding such as pAb421 or the carboxy-terminal peptide cannot further activate p53 in the presence of Ref-1. Conversely, although stimulation of oxidized p53 by Ref-1 cannot be enhanced further by either antibody or peptide, these effectors can still further stimulate p53 that has been reduced by DTT. Second, in the presence of DTT, Ref-1

stimulates DNA binding by full-length but not truncated p53 Δ 30. While these data argue convincingly for a redox-independent function of Ref-1, final confirmation of this assumption awaits testing redox-defective mutants of Ref-1. The data described here imply that although reduction of p53 is essential for its activation, it is not sufficient. The fact that p53 Δ 30 cannot be appreciably stimulated beyond what is achieved by reduction suggests that the second level of control of p53 DNA binding is contributed by the inhibitory carboxyl terminus. Both pAb421 and carboxy-terminal fragments or peptides have been shown previously to stimulate p53 most likely by relieving inhibition by the carboxyl terminus. Ref-1 may be the physiological counterpart of these artificial effectors, a likelihood supported by the fact that it is at least two orders of magnitude more effective than they are in stimulating p53. It is probable that a critical feature of carboxy-terminal peptides for stimulation of p53 is their highly basic nature. Likewise, the amino terminus of Ref-1 is unusually basic: Of the amino-terminal 100 amino acids, 21 are basic residues, supporting the suggestion that the nonredox component of Ref-1 is related to the effect of basic peptides on p53 DNA binding.

Ref-1 and p53 can functionally interact in vivo

Transient transfection assays in p53 null cell lines revealed a significant stimulatory effect of Ref-1 on the ability of cotransfected p53 to activate promoters that are likely p53 targets in cells. The observation that Ref-1 did not stimulate p53 Δ 30 in vivo supports the assumption that this carboxy-terminally truncated form of p53 is modulated only by redox, that is, once reduced, it is fully active and cannot be stimulated further by Ref-1. This is significant because (1) it supports our conclusion that there is a redox-independent effect of Ref-1, and (2) at the highest level of p53 Δ 30 tested its activation equals that of wild-type p53 + Ref-1, indicating that in the absence of Ref-1, a comparable amount of wild-type p53 is not fully activated.

It is acknowledged that Ref-1 stimulation of p53 in vivo is not as dramatic as in in vitro DNA-binding assays, which may be attributable in part to the fact that both H1299 and Saos2 cells contain significant quantities of endogenous Ref-1 (data not shown). Ref-1 is a fairly abundant protein and clearly an extremely important one, as it is necessary for cell and organismic viability (Xanthoudakis et al. 1996). Thus, in most situations Ref-1 is not limiting and the endogenous Ref-1 would be sufficient to activate levels of p53 such as those produced by transfecting moderate amounts of p53. However, what we have shown is that Ref-1 can be limiting in cells; that is, when we transfect in a large quantity of p53, more Ref-1 is required to get full activation. The fact that overexpressed Ref-1 can elicit further activation by p53 in cells is very encouraging. Although we have determined that Ref-1 levels are not affected by DNA damage in RKO cells (data not shown), a strong inducer of Ref-1 was shown to be cellular hypoxia (Yao et al.

1994). This is relevant to the exciting finding that hypoxia is also a critical inducer of wild-type p53 in tumors leading to apoptosis (Graeber et al. 1996).

How does p53 escape Ref-1 in normal cells?

The fact that Ref-1 has such a powerful stimulatory effect on p53 in vitro poses another question. Because we were able to first identify Ref-1 as an effective activator of latent p53 in a crude fraction from a HeLa nuclear extract, how then is latent p53 "protected" from an effectors such as Ref-1 in cells? Although the answer is not yet known we can speculate as follows:

1. *The two proteins are physically separated in cells until p53 function is required.* Although both proteins are predominantly nuclear, in most cases direct immunofluorescence analysis indicates that Ref-1 is located at the periphery of the nucleus (Xanthoudakis et al. 1992), which is quite different from the more global nuclear staining patterns of p53. It will thus be interesting to examine the distribution of both proteins after either oxidative stress or DNA damage to see if they colocalize.
2. *Ref-1 might be negatively regulated until needed.* It is possible that Ref-1 function may itself be subjected to post-translational regulation. It is known that when mammalian cells are treated with oxidizing agents they respond with increased synthesis and activity of reducing enzymes and antioxidants such as glutathione and thioredoxin (Meister and Anderson 1983; Holmgren 1984). Xanthoudakis et al. (1992) have also shown that Ref-1 activity is stimulated/regenerated by thioredoxin/thioredoxin reductase/NADPH. It is therefore possible that Ref-1 is sensitive to the redox state of the cell.
3. *p53 protein in cells exists in a physical form that cannot be activated by Ref-1 until the necessary stimulus occurs.* In this scenario p53 would not be able to be stimulated by Ref-1 unless it were first acted upon by other cellular factors. Immunoaffinity purification of p53, even in its latent form, would "unlock" this inaccessible conformation and allow it to be stimulated by Ref-1.

Ref-1 may provide a link between DNA repair and transcriptional activation by p53

The DNA repair function of Ref-1 was discovered by its sequence identity to the class II hydrolytic A/P endonuclease DNA repair protein APE/APEX/HAP-1. In addition to its A/P endonuclease activity, Ref-1 was shown to possess a 3' phosphodiesterase activity capable of removing lesions blocking the 3' OH of DNA strand breaks that are generated by ionizing radiation (Chen et al. 1991). It was reported that down-regulation of Ref-1 leads to increased sensitivity to DNA damaging agents in cells (Walker et al. 1994). Ref-1 is thus a multifunctional protein, housing within its sequences both DNA repair functions and the ability to regulate DNA-binding

activity of AP-1, as well as additional transcription factors; therefore, it is unique in being a single polypeptide that links the process of DNA repair with transcriptional regulation. This feature of Ref-1 is particularly relevant in the case of p53, itself a transcription factor that has been shown to respond to DNA damage. It is exciting to speculate that the two domains of Ref-1 may act coordinately to bring about DNA repair as well as to stimulate the DNA-binding activity of latent p53 in the cell during oxidative stress. In the case of hypoxia the net effect would be a severalfold augmentation in the cellular response to adverse oxidative trauma, both in terms of magnitude and rapidity of response. Such a strategy would be invaluable in minimizing cellular damage caused by potentially harmful oxidative conditions. Interactions between Ref-1 and p53 could ensure rapid and efficient cell-cycle arrest and concomitant DNA repair.

Ref-1 is not the only DNA repair factor that can affect or interact with p53. TFIH is a dual transcription repair factor (for review, see Drapkin et al. 1994) that consists of several polypeptides, at least three of which can bind to p53. The p62 polypeptide component of TFIH binds to the amino terminus of p53, whereas the XP-B and XP-D constituents were reported to interact with the p53 carboxyl terminus (Xiao et al. 1994; Wang et al. 1995; Leveillard et al. 1996). Our finding that another DNA repair factor, Ref-1, activates latent p53 underscores the possibility that p53 may be poised such that it can directly transduce signals from DNA repair processes to genes that control the cell cycle and apoptosis.

Materials and methods

Purification of p53 proteins

Flu peptide-tagged human wild-type, full-length p53 and p53 Δ 30 constructs were generated by PCR using oligonucleotides corresponding to fixed 5' and variable 3' end points (1–393 and 1–363) of the p53 coding sequence resulting in the second residue of p53 affixed to the flu sequence: MGYPYDVPDYA. Sf-21 cells were infected with baculoviruses expressing flu-tagged p53 or p53 Δ 30 and harvested 48 hr postinfection, and p53 proteins were immunopurified (Jayaraman and Prives 1995). Infected insect cell extracts were passed over columns packed with protein A–Sepharose cross-linked with either pAb421 (for active p53) or mAb12.CA5 (for latent p53) antibody. Elution was performed using either the flu epitope peptide YPYDVPDYA (for latent p53 and flu-tagged p53 Δ 30) or the 421 epitope peptide KKGQSTSRHKK (for active p53). Oxidized p53 was prepared similarly except that the flu peptide was used for the elution step; and extraction, elution and dialysis were done in buffers lacking DTT. The human p53 carboxyl terminus fragment (311–393) was a generous gift from N. Pavletich (Memorial Sloan-Kettering Cancer Center, New York, NY).

DNA binding

EMSA was carried out as described previously (Jayaraman and Prives 1995). The oligonucleotide probe containing the GADD45 site is as follows: 5'-AATTCTCGAGCAGAACATGTCTAAGCATGCTGGGCTCGAG-3'. The probe was labeled by the Klenow fragment of *Escherichia coli* DNA polymerase. Reaction mixtures contained 8 μ l of 5 $\times$ EMSA buffer [100 mM

HEPES (pH 7.9), 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl₂, 2 μ l of 40 mM spermidine, 2 μ l of 10 mM DTT, 2 μ l of 0.5% NP-40, 2 μ l of 60 mg/ml double-stranded poly[d(I-C)], 4 μ l of bovine serum albumin (1 mg/ml), ³²P-labeled probe DNA (3 ng), proteins and antibodies as indicated, and water in a total volume of 40 μ l. Reaction mixtures were incubated at room temperature for 30 min; 20 μ l of each reaction mixture was then loaded onto a native 4% polyacrylamide gel containing 0.5 $\times$ Tris–borate-EDTA (TBE) buffer, 1 mM EDTA, and 0.05% NP-40 and electrophoresed in 0.5% TBE at 4°C at 200–250 V for 2 hr. DNA–protein complexes were quantified by PhosphorImaging using Image-Quant software.

In vitro transcription

The fractions used to provide the RNA polymerase II and related transcription factors were prepared and used as described previously (Farmer et al. 1992). The flowthrough from the P11 column was passed over a DEAE–cellulose (DE52, Whatman) column and step-eluted with 0.035 M KCl. This fraction contained the TFIIA. The P11 0.5 M KCl fraction (P11.5) was passed over a double-stranded DNA Sepharose 4B column, the flowthrough of which provided TFIIB, TFIIE, TFIIIF, and RNA polymerase (Prwyes and Roeder 1987). The P11 0.85 M KCl fraction was used as a source of TFIID. The *in vitro* transcription reaction, using pretested amounts of the three fractions, contained as the test template (pWAF–MLT) a G-less cassette vector containing the WAF-1 p53 binding site cloned upstream of the adenovirus major late promoter (generously provided by Sheau-Yann Shieh, Columbia University, New York, NY). The reference template (pML cassette) was the same construct but lacking the WAF-1 site. Reaction mixtures contained 5 mM MgCl₂, 10 mM HEPES, 0.5 mM ATP, 0.5 mM UTP, 20 μ M CTP, 0.25 mM O-METHYL GTP, 5 mM creatine phosphate, 50 units of RNase T1, 20 units of RNasin, 5 μ Ci of [³²P]CTP, 75 ng each of reference and test template, proteins as indicated, nuclear extract fractions, and water in a total volume of 25 μ l. Preincubation complexes were allowed to form on templates at 30°C for 10 min in reaction mixtures equivalent to that described above, except that the salts and NTPs were absent, after which these components were added and the reaction mixture incubated for an additional 50 min. Reactions were stopped by using transcription stop buffer (50 mM Tris-HCl at pH 7.5, 1% SDS, 5 mM EDTA, and 20 μ g/ml of tRNA, phenol/chloroform/isoamyl alcohol extracted, ethanol precipitated, and the pellets resuspended in 10 μ l of 80% formamide/10 mM EDTA. The samples were heated for 5 min at 95°C, loaded onto a 4% polyacrylamide/urea gel and electrophoresed at 150 V for 1 hr.

Purification of Ref-1

Nuclear extracts (7 mg/ml), prepared from ~50 liters of HeLa cells according to the procedure of Dignam et al. (1983) and dialyzed into BC100 buffer (100 mM KCl, 20 mM Tris at pH 8.0, 0.2 mM EDTA, and 20% glycerol), were aliquoted, frozen, and stored at –80°C. Protein purification was carried out at 4°C, and samples were kept on ice. All chromatographic steps were performed by FPLC, except for the Superdex-75 fractionation, which was performed on the SMART fractionation system (Pharmacia). HeLa nuclear extract (300 mg) was loaded onto a 20-ml phosphocellulose P11 column and washed with two column volumes of BC100. The bound protein was eluted stepwise with BC100 buffer containing 0.3, 0.5, and 0.85 M KCl. In this and all subsequent chromatographic steps, protein concentration was monitored by continuous UV absorption at 280 nm. The p53 stimulatory fractions from the P11 column (as assayed

by gel retardation analysis) were pooled, dialyzed against BC100 buffer, and loaded onto a 10-ml DE52 column equilibrated in the same buffer. The flowthrough fraction, which contained most of the stimulatory activity, was applied directly onto a 5-ml heparin-agarose column, washed with three column volumes of BC100 buffer, and eluted stepwise with that buffer containing 0.5 and 1 M KCl. The 0.5 M fraction, which contained the stimulatory activity, was dialyzed against BC100 buffer and fractionated further by two consecutive rounds of mono-S chromatography. The stimulatory activity was eluted using a linear gradient of 0.1–1 M KCl in BC100 buffer, and peak fractions were pooled and dialyzed before gel filtration chromatography on a Superdex-75 column. The protein composition of different column fractions was analyzed on 10% SDS-polyacrylamide gels and silver-stained for visualization of bands. The protein concentration of pooled fractions from individual columns was estimated using the Bio-Rad protein determination kit.

Bacterially expressed recombinant His-tagged Ref-1 and carboxy-terminally deleted Ref-1 proteins were prepared as described by Xanthoudakis et al. (1992) by binding to and elution from Ni-agarose beads (Qiagen).

Transfection assays

H1299 and Saos-2 cells (American Type Culture Collection) were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). p53, p53Δ30 (kindly provided by X. Chen, Medical College of Georgia, Augusta), and Ref-1 (S. Xanthoudakis and T. Curran, unpubl.) cDNAs were under the control of the cytomegalovirus (CMV) promoter. The reporter construct used was pGL-cyclin G-luc, containing 1.48 kb of the 5' region of the rat cyclin G gene (kindly given to us by M. Oren, Weizmann Institute of Science, Rehovot, Israel). Prior to transfection, cells were seeded at 0.6×10^6 cells per 60-mm-diam. dish for luciferase assays. Cells were transfected by the calcium phosphate method and the precipitate left on the cells for 5 hr, after which cells were plated in fresh DMEM supplemented with 10% FBS. Luciferase assays were performed 18–24 hr later. When appropriate, DNA of the parental vector was included to keep the total amount of transfected DNA constant in each sample. Luciferase assays were essentially as described by Friedlander et al. (1996). Each transfection experiment was performed in triplicate cultures.

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Identification of redox/repair protein Ref-1 as a potent activator of p53.

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