Oncogene (2000) 19, 5259–5269
© 2000 Macmillan Publishers Ltd All rights reserved 0950–9232/00 $15.00
www.nature.com/onc

p53 mediates Bcl-2 phosphorylation and apoptosis via activation of the Cdc42/JNK1 pathway

Anju Thomas[1], Theresa Giesler[1,2] and Eileen White*[,1,2,3,4]

[1]*Center Adanced Biotechnology and Medicine, Rutgers University, 679 Hoes Lane, Room 140, Piscataway, New Jersey, NJ 08854, USA;* [2]*Howard Hughes Medical Institute, Rutgers University, 679 Hoes Lane, Room 140, Piscataway, New Jersey, NJ 08854, USA;* [3]*Cancer Institute of New Jersey, Rutgers University, 679 Hoes Lane, Room 140, Piscataway, New Jersey, NJ 08854, USA;* [4]*Department of Molecular Biology and Biochemistry, Rutgers University, 679 Hoes Lane, Room 140, Piscataway, New Jersey, NJ 08854, USA*

A member of the small G protein family, *cdc42*, was isolated from a screen undertaken to identify p53-inducible genes during apoptosis in primary baby rat kidney (BRK) cells transformed with E1A and a temperature-sensitive mutant p53 using a PCR-based subtractive hybridization method. Cdc42 is a GTPase that belongs to the Rho/Rac subfamily of Ras-like GTPases. In response to external stimuli, Cdc42 is known to transduce signals to regulate the organization of the actin cytoskeleton, induce DNA synthesis in quiescent fibroblasts, and promote apoptosis in neuronal and immune cells. In this study, we have demonstrated that *cdc42* mRNA and protein were up-regulated in the presence of wild-type p53 in BRK cells, followed by cytoplasmic to plasma membrane translocation of Cdc42. Overexpression of Cdc42 in the presence of a dominant-negative mutant p53 induced apoptosis rapidly, indicating that Cdc42 functions downstream of p53. Furthermore, stable expression of a dominant-negative mutant of Cdc42 partially inhibited p53-mediated apoptosis. The Bcl-2 family members Bcl-x_L, and the adenovirus protein E1B 19K, inhibited Cdc42-mediated apoptosis, whereas Bcl-2 did not. We provide evidence that PAK1 and JNK1 may play a role downstream of Cdc42 to transduce its apoptotic signal. Cdc42/PAK1 activates JNK1-induced phosphorylation of Bcl-2, thereby inactivating its function, and that a phosphorylation resistant mutant (Bcl-2S70,87A,T56,74A) gains the ability to inhibit Cdc42- and p53-mediated apoptosis. Thus, one mechanism by which p53 promotes apoptosis is through activation of Cdc42 and inactivation of Bcl-2. *Oncogene* (2000) **19,** 5259–5269.

Keywords: Cdc42; p53; Bcl-2; JNK1; PAK1; apoptosis

Introduction

The tumor suppressor p53 is an important component of the cellular machinery that responds to genotoxic stresses, particularly DNA damage, to maintain genomic integrity by arresting the cell cycle or by inducing apoptosis (Ko and Prives, 1996; Levine, 1997). In normal cells, the tumor suppressor p53 protein is found in low levels because of its short half-life (Levine, 1997). In response to several types of DNA damage, including double-stranded breaks formed by γ-irradiation and DNA-repair intermediates formed by UV irradiation, p53 levels are rapidly induced and its transcriptional activity is activated. Although not always the case, there is substantial evidence that much of the biological activity of p53 is achieved through its ability to regulate gene transcription (Buckbinder *et al*., 1995; El-Deiry *et al*., 1993; Miyashita *et al*., 1994a; Miyashita and Reed, 1995; Owen-Schaub *et al*., 1995).

The *bax* gene is known to be transcriptionally regulated by p53 during induction of apoptosis (Buckbinder *et al*., 1995; Miyashita and Reed, 1995). Bax is a pro-apoptotic Bcl-2 family member that binds to the anti-apoptotic Bcl-2 protein, and can antagonize its function to block apoptosis (Oltvai *et al*., 1993). Induction of Bax also promotes cytochrome *c* release from the mitochondria, which is inhibited by anti-apoptotic Bcl-2 family members (Rossé *et al*., 1998), therefore, *bax* up-regulation is one mechanism whereby p53 induces apoptosis. The biochemical mechanism of Bcl-2 and Bax function, however, remains elusive. Structural studies of Bcl-2 family members have demonstrated similarities to pore-forming domains of bacterial toxins, which has suggested a role in modulation of membrane function (Chou *et al*., 1999; McDonnell *et al*., 1999; Minn *et al*., 1997; Sattler *et al*., 1997; Schendel *et al*., 1999).

Other genes regulated by p53 during apoptosis are *IGF-BP3* (Buckbinder *et al*., 1995) and *Fas/Apo-1* (Owen-Schaub *et al*., 1995). IGF-BP3 has been shown to block IGF-induced signaling by binding to IGF and preventing its binding to its receptor, and inhibiting IGF activity promotes apoptosis in cells. It has also been reported that p53 can induce the expression of the *Fas/Apo-1* gene (Owen-Schaub *et al*., 1995). The cell surface receptor, Fas, mediates apoptosis through a cascade of signaling molecules (Nagata, 1997). Therefore, inducing apoptosis through the Fas pathway would represent another mechanism by which p53 can promote cell death.

We have previously characterized a transformed BRK epithelial cell line (p53A cells) expressing the adenovirus E1A protein and the temperature-sensitive mutant of p53(va1135) (Debbas and White, 1993). The temperature sensitive p53 mutant is in the mutant conformation at the nonpermissive temperature of 38.5°C and is in the wild-type conformation at the permissive temperature of 32°C. As reported pre-

*Correspondence: E White, CABM/HHMI, 679 Hoes Lane, Room 140, Piscataway, New Jersey, NJ 08854, USA
Received 26 June 2000; revised 22 August 2000; accepted 30 August 2000

viously, p53A cells are transformed and proliferate at 38.5°C and at 32°C undergo p53-dependent cell death by apoptosis (Debbas and White, 1993; Sabbatini *et al*., 1995b; Thomas and White, 1998). Previous studies have shown that *bax* is up-regulated during apoptosis in p53A cells (Han *et al*., 1996; Sabbatini *et al*., 1995b). However, Bax induction may not be sufficient for p53-dependent apoptosis. Bax null fibroblasts expressing the adenovirus E1A protein are still able to undergo p53-dependent apoptosis, although the response is less robust (McCurrach *et al*., 1997). Furthermore, BRK cells expressing E1A and a mutant of p53 with deletion of the proline-rich region which retains the ability to up-regulate *bax* expression, is defective for apoptosis induction (Sakamuro *et al*., 1997). Thus, *bax* induction is not sufficient for apoptosis by p53. As a transcriptionally crippled p53 mutant is not capable of inducing apoptosis (Sabbatini *et al*., 1995b), yet unidentified genes that are transcriptionally regulated by p53 must play a role in implementing apoptosis. In light of this evidence, we screened a subtractive cDNA library for p53-inducible genes during p53-dependent apoptosis.

Cdc42, a member of the Rho/Rac family of small G proteins was isolated from this screen. Members of the Rho/Rac family of GTPases are signal mediators that regulate cytoskeletal changes, transcription, cell cycle control, and apoptosis (Hall, 1998; Johnson, 1999; Van Aelst and D'Souza-Schorey, 1997). The small GTPases regulate these molecular mechanisms by cycling from the inactive GDP-bound state to the active GTP-bound form. The binding to GTP/GDP is regulated by interaction of the GTPases with GEFs, GDP dissociation inhibitors, and GAPs (Hall, 1998; Johnson, 1999; Van Aelst and D'Souza-Schorey, 1997).

Cdc42 regulates signaling by binding to downstream effector proteins (Tapon and Hall, 1997) mainly through its CRIB domain (Burbelo *et al*., 1995). Reports have shown that Cdc42 and its downstream effectors, PAK1-3 and JNK/SAPK, are involved in stress-activated apoptosis (Bazenet *et al*., 1998; Chuang *et al*., 1997). The downstream events or the phosphorylation targets that promote stress-activated apoptosis are not known. Expression of the activated form of Cdc42 in Jurkat T lymphocytes (Chuang *et al*., 1997) as well as in rat sympathetic neurons (Bazenet *et al*., 1998) induce an apoptotic response. In this report, we have shown that p53 can indirectly activate the Cdc42 GTPase to induce apoptosis. Cdc42 recruits PAK1 and activates JNK1 to phosphorylate, and consequently inactivate, the anti-apoptotic Bcl-2 protein allowing p53-dependent apoptosis.

Results

Activation and localization of Cdc42 in the presence of wild-type p53

In order to identify novel p53-inducible genes during apoptosis, a subtractive cDNA library was made from primary BRK epithelial cell line, p53A, expressing the adenovirus E1A protein and a temperature-sensitive p53(val135) mutant (Debbas and White, 1993). As previously reported, p53A cells express the temperature sensitive p53(val135) mutant in which p53 is in the wild-type conformation at the permissive temperature of 32°C and in the mutant conformation at the nonpermissive temperature of 38.5°C (Debbas and White, 1993). Previous studies have shown that p53A cells incubated at the permissive temperature induced high levels of wild-type p53 protein and undergo massive p53-dependent apoptosis (Debbas and White, 1993; Han *et al*., 1996; Sabbatini *et al*., 1995a,b; Thomas and White, 1998). A subtractive cDNA library was made from p53A cells expressing mutant p53 at 38.5°C and from cells expressing wild-type p53 at 32°C. Our aim was to identify genes that are transcriptionally up-regulated by wild-type p53 during apoptosis.

One of the genes identified from this library screen was *cdc42*, a member of the small G protein family. Northern blot analysis of *cdc42* in p53A cells incubated at 32°C for 4 h showed a slight increase in *cdc42* message when wild-type p53 was induced (Figure 1a). $p21^{WAF1}$, a known p53-inducible gene was up-regulated at this time interval (Figure 1a). Competitive PCR analysis on cDNA from p53A cells incubated at 38.5°C versus cells at 32°C for 4 h showed a twofold increase in *cdc42* message (data not shown). The small increase of *cdc42* RNA was not observed when cycloheximide, a protein synthesis inhibitor, was present when p53 was induced (Figure 1a), which indicated that transcriptional regulation of *cdc42* by p53 was probably not direct.

We then examined Cdc42 protein expression in p53A cells incubated at the permissive temperature for various time intervals. Up-regulation of endogenous Cdc42 protein was observed in p53A cells (Figure 1b) when wild-type p53 was induced (Debbas and White, 1993). In contrast, Cdc42 protein was not induced in BRK cells expressing E1A and 22/23p53(val135) (Figure 1b), a transcriptionally defective mutant of p53 (Sabbatini *et al*., 1995b). Thus, *cdc42* RNA and protein are induced by transcriptionally functional p53.

Since G-proteins are known to become membrane-associated upon activation (Boivin and Beliveau, 1995; Johnson, 1999), we examined whether Cdc42 was present in the soluble or insoluble fractions during apoptosis. Soluble and insoluble lysates from p53A cells undergoing apoptosis during various time intervals were electrophoresed on SDS–PAGE. Cdc42 protein appears to shift from soluble to the insoluble fraction as cell death progress (Figure 1c, upper panel). There is less Cdc42 protein in Figure 1c, day 4 (soluble/insoluble) than in Figure 1b, day 4 because these are independent experiments using two different lysis buffers, therefore, the results may have slight variations. These results suggest that Cdc42 may localize to membranes or cytoskeletal structures when apoptosis is induced in cells. We next wanted to determine whether Cdc42 was present in its active GTP-bound form in the presence of wild-type p53. To determine the state of Cdc42, we took advantage of an affinity precipitation assay using the regulatory domain (residues 67–150) of PAK1 (Benard *et al*., 1999). The protein-binding domain of PAK1 functions as a potent affinity ligand for the active small G proteins, Cdc42 and Rac. Soluble lysates from the p53A cells undergoing apoptosis were used to affinity precipitate Cdc42-GTP. The precipitates were electrophoresed on SDS–PAGE and blotted with a monoclonal antibody specific for Cdc42. The presence of Cdc42 in the precipitates indicates that it is in the active GTP-bound form.

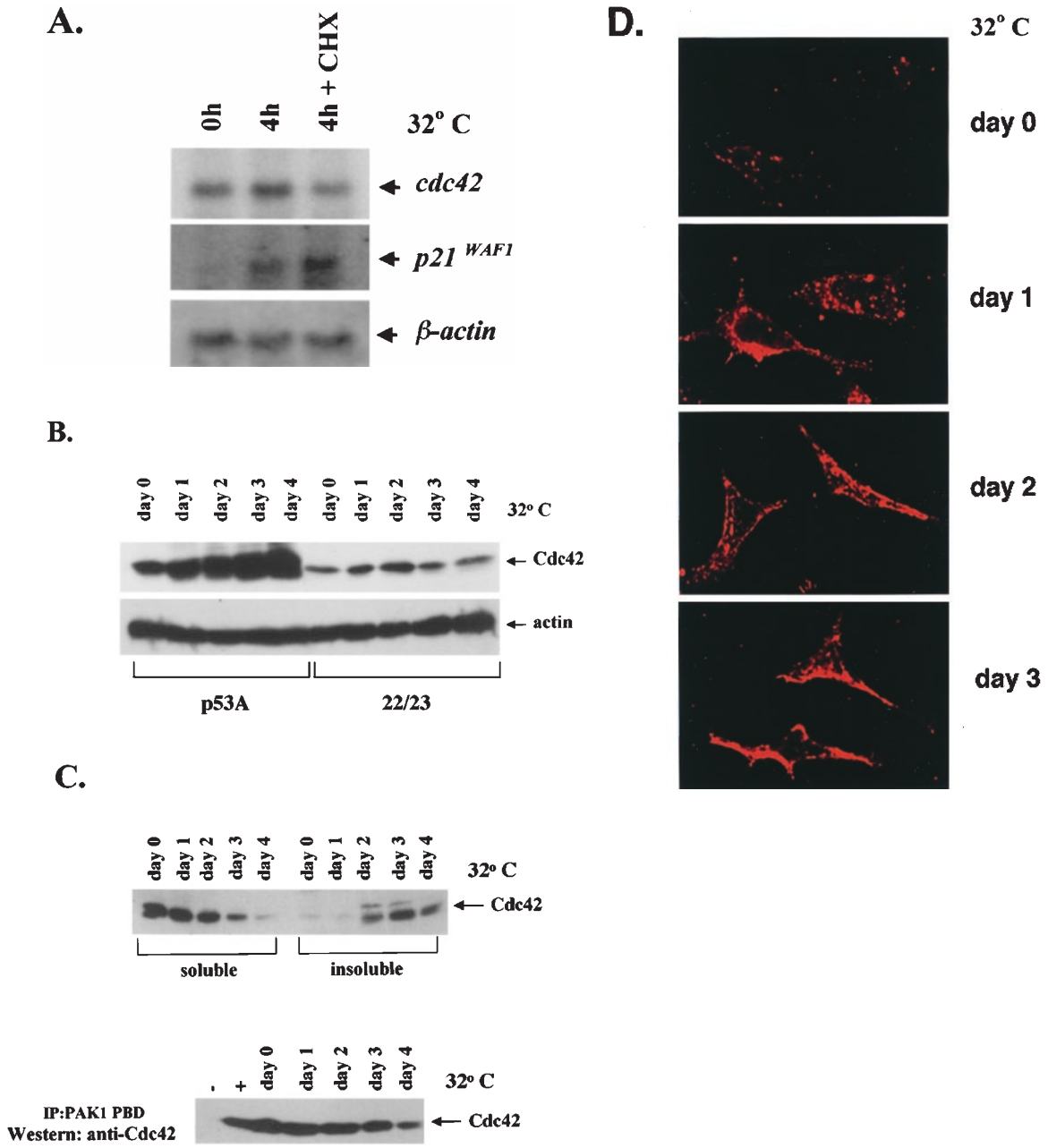

Figure 1 Cdc42 protein is up-regulated and translocated to the plasma membrane during p53-dependent apoptosis. (**a**) Northern blot analysis of p53A cells incubated at 38.5°C (0 h), and at 32°C for 4 h and 4 h plus 20 μg/ml cycloheximide (CHX). ^{32}P-labeled *cdc42*, *p21^{WAF1}*, and *actin* cDNAs were used as probes. (**b**) Western blot analysis of Cdc42 and actin in p53A and 22/23 cells incubated at 38.5°C (day 0) and at 32°C from day 1 to day 4. (**c**) Western blot of Cdc42 in soluble and insoluble lysates from p53A cells incubated at the nonpermissive temperature (day 0) and at the permissive temperature from day 1 to day 4 (upper panel). The soluble lysates were used for affinity precipitation/Western blot of Cdc42 in p53A cells at 38.5°C (day 0) and at 32°C from day 1 to day 4 (lower panel). The protein binding domain (residues 67–150) of PAK1 was used as an affinity ligand for GTP-bound Cdc42 (Benard *et al*., 1999). Negative (−) and positive (+) control represent HeLa extracts loaded with GDP or GTPγS, respectively. (**d**) Indirect immunofluorescence of Cdc42 in p53A incubated at 38.5°C (day 0) and at 32°C from day 1 to day 3

Surprisingly, our data shows that Cdc42 was in the active GTP-bound state (Figure 1c, lower panel), even in the presence of mutant p53. These results suggested that p53 expression might not affect the proportion of Cdc42 in the GTP form, but did lead to an increase in total Cdc42, which may influence total amount of Cdc42-GTP.

Previous studies have shown that Cdc42 proteins are mostly membrane bound in the presence of GTPγS through their post-translational modifications (for example, farnesylation of the CAAX motif) and are localized to either internal membranes or plasma membranes (Boivin and Beliveau, 1995). Therefore, we next wanted to determine the endogenous localization of Cdc42 during induction of wild-type p53. At the permissive temperature, Cdc42 appeared to translocate to the plasma membrane as cells undergo p53-mediated apoptosis (Figure 1d). These results suggested that activated Cdc42 in the presence of wild-type p53 underwent post-translational modifications, which may promote translocation of Cdc42 to the plasma membrane. In addition, we have examined Cdc42 translocation in p53A cells stably expressing E1B 19K, an adenovirus protein that inhibits p53-mediated

apoptosis (Debbas and White, 1993). Translocation of Cdc42 to the plasma membrane was delayed in p53A cells expressing E1B 19K (data not shown). However, prolonged incubation of 19K expressing cells at 32°C eventually induced translocation of Cdc42 to the plasma membrane (data not shown). These results suggest that Cdc42 translocation may be due to p53 activation and not a consequence of apoptosis.

Overexpression of Cdc42 promotes apoptosis, bypassing the requirement for wild-type p53

To investigate further the role of Cdc42 in p53-mediated apoptosis, we examined whether transient expression of Cdc42 can promote apoptosis in the absence of wild-type p53. For this assay, we used several mutants of Cdc42. As mentioned previously, Cdc42 is a GTPase where it is active when bound to GTP and inactive when bound to GDP. Therefore, we used two activated mutants of Cdc42, Cdc42V12 (valine substituted for glycine 12) and Cdc42L61 (leucine substituted for glutamine 61), and a dominant-negative mutant, Cdc42N17 (threonine substituted for asparagine 17), to determine its effects on cell viability. The activated Cdc42 mutants are constitutively GTP-bound due to their inability to hydrolyze bound GTP. In contrast, the dominant negative Cdc42 mutant is constitutively GDP-bound. Western blot analysis of p53A cells transiently transfected with Cdc42 mutant reveals equivalent expression (Figure 2a, upper panel). Our results show that wild-type and the activated mutants of Cdc42 induced apoptosis in p53A cells at 38.5°C (Figure 2a, lower panel). In contrast, the dominant-negative mutant of Cdc42 did not induce apoptosis (Figure 2a, lower panel). These results suggested that overexpression of Cdc42 did induce cell death, bypassing the requirement for p53.

Since Cdc42 lies downstream of p53, we wanted to determine whether dominant-negative Cdc42 could inhibit p53-mediated cell death. Stable clones of p53A cells expressing dominant-negative Cdc42 or cells expressing vector alone were assessed for cell viability at the permissive temperature. Thirteen stable clones expressing various levels of Cdc42N17 and four vector control cell lines were established (Figure 2b). As

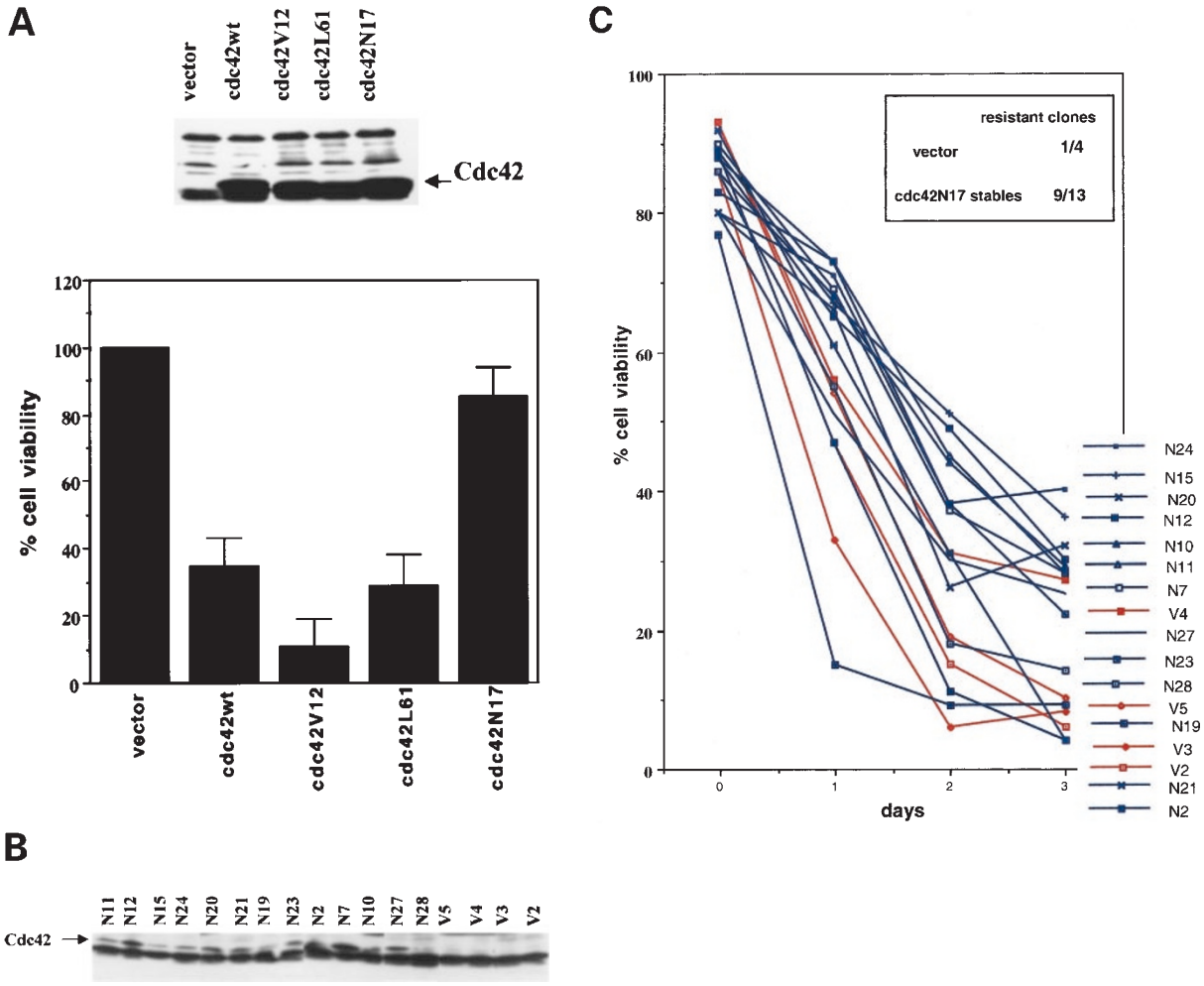

Figure 2 Overexpression of Cdc42 induces cell death. (**a**) Western blot analysis of Cdc42 in p53A cells transiently transfected with vector alone, pRK5cdc42wt, pCGcdc42V12, pRK5cdc42L61, pRK5cdc42N17, and pcDNA3.1lacZ and cell viability was determined 24 h post-transfection by staining for β-galactosidase activity (blue cells). (**b**) Western blot analysis of Cdc42 in stable clones of p53A cells expressing dominant-negative Cdc42N17 mutant. (**c**) Cell viability measured by trypan blue exclusion in stable clones of p53A cells transfected with the empty vector or the dominant-negative Cdc42N17 mutant expression plasmid incubated at 38.5°C (day 0) and at 32°C from day 1 to day 3. Vector controls are shown in red, whereas dominant-negative Cdc42N17 expressing cells is shown in blue

shown in Figure 2c, nine out of 13 dominant-negative Cdc42 expressing stable clones were more resistant to p53-induced apoptosis. One out of the four vector controls was resistant to p53-dependent apoptosis. This variability may be due to clonal variation or genomic instability. A complete inhibition of cell death was not observed, probably due to the ability of p53 to induce apoptosis through other cascades, such as up-regulation of *bax*. Our results suggest that Cdc42 may be involved in one of the pathways in which p53 induces cell death.

PAK1 plays a role in p53-mediated apoptosis

The Bcl-2 family members are known inhibitors of apoptosis (Gross *et al*., 1999; Korsmeyer, 1995; White, 1996). Therefore, we wanted to determine whether these inhibitors could block Cdc42-induced cell death. Comparable transient expression of the apoptosis inhibitors, E1B 19K, Bcl-X_L and Bcl-2, in p53A cells was detected (Figure 3a, upper panel). Transient expression of Cdc42wt and Cdc42V12 in p53A cells induced efficient apoptosis at 38.5°C, whereas the Bcl-2 homologs, adenovirus protein E1B 19K and Bcl-x_L effectively inhibited Cdc42wt- and Cdc42V12-mediated apoptosis (Figure 3a, lower panel). Surprisingly, Bcl-2 did not block this cell death (Figure 3a, lower panel).

Proteins that bind to both Cdc42 and Rac include the serine/threonine kinases PAK1-3 (Dharmawardhane *et al*., 1997; Sells and Chernoff, 1997; Zhao *et al*., 1998). Binding of Rac- and Cdc42-GTP to PAKs leads to autophosphorylation and activation of this kinase, thus enabling it to phosphorylate substrates on serine/threonine residues. In order to determine whether PAK1 plays a role in mediating apoptotic signal from Cdc42, we examined whether a dominant-negative form of PAK1 can block Cdc42wt- and Cdc42V12-mediated apoptosis. The dominant-negative form of PAK1 was constructed by substituting the active lysine 299 for an arginine in the kinase domain (Tang *et al*., 1997). In addition to detecting the 58 kDa PAK1 band, Western blot analysis of ectopically expressed dominant-negative PAK1 revealed a background band of approximately 26 kDa (Figure 3a, upper panel). Transient expression of dominant-negative PAK1 did indeed reduce Cdc42wt- and Cdc42V12-mediated apoptosis at 38.5°C (Figure 3a, lower panel). Our results suggested that PAK1 might play a role in the apoptotic signal transduction cascade induced by Cdc42.

To confirm this finding, we next examined whether p53A cells expressing a dominant-negative form of PAK1 can block p53-mediated apoptosis at the permissive temperature. Seven stable clones of p53A cells expressing dominant-negative PAK1 and three vector controls (Figure 3b) were isolated and assessed for their ability to block p53-mediated apoptosis. Six out of seven dominant-negative PAK1 stable cell lines were significantly more resistant to p53-mediated apoptosis than all of the three vector control cell lines (Figure 3c). Our data indicated that dominant-negative PAK1 partially blocked p53-dependent apoptosis at the permissive temperature (Figure 3c). Again, the incomplete inhibition of p53-mediated apoptosis by dominant-negative PAK1 was probably due to activation of more than one apoptotic pathway by p53 or by Cdc42. Our results suggested that Cdc42 might induce apoptosis in p53A cells through PAK1 kinase.

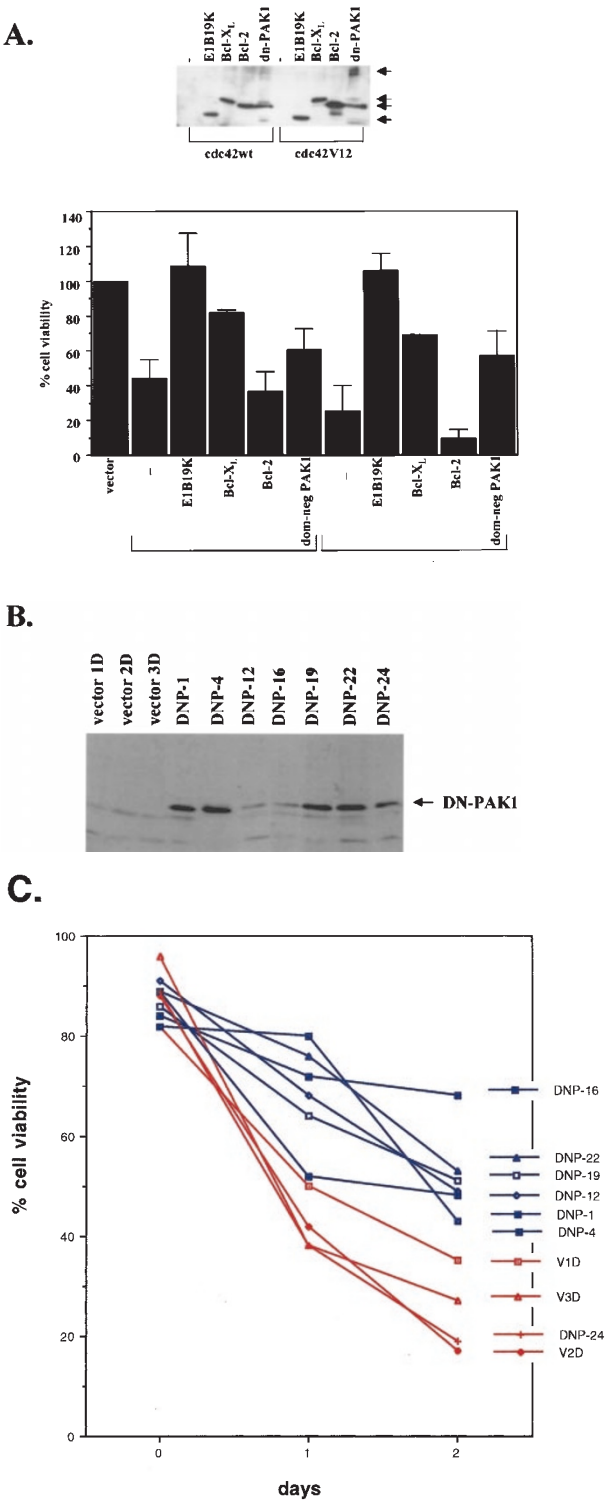

Figure 3 Cdc42 induces apoptosis through PAK1. (**a**) Cotransfection of p53A cells with vector alone, pRK5cdc42wt, pCGcdc42V12, pcDNA3.1E1B19K, pcDNA3Bcl-x_L, pcDNA3.1Bcl-2, pcDN3dom-negPAK1, and pcDNA3.1lacZ. Western blot analysis of transfected cells (upper panel). Twenty-four hours post-transfection, cell viability was assessed by staining for β-galactosidase activity (lower panel). (**b**) Western blot analysis of PAK1 in stable clones of p53A cells expressing dominant-negative PAK1 mutant. (**c**) Cell viability as measured by trypan blue exclusion in stable clones of p53A cells transfected with the empty vector or the dominant-negative PAK1 mutant expression plasmid incubated at 38.5°C (day 0) and at 32°C from day 1 to day 2. Vector controls are shown in red, whereas dominant-negative PAK1 expressing cells is shown in blue

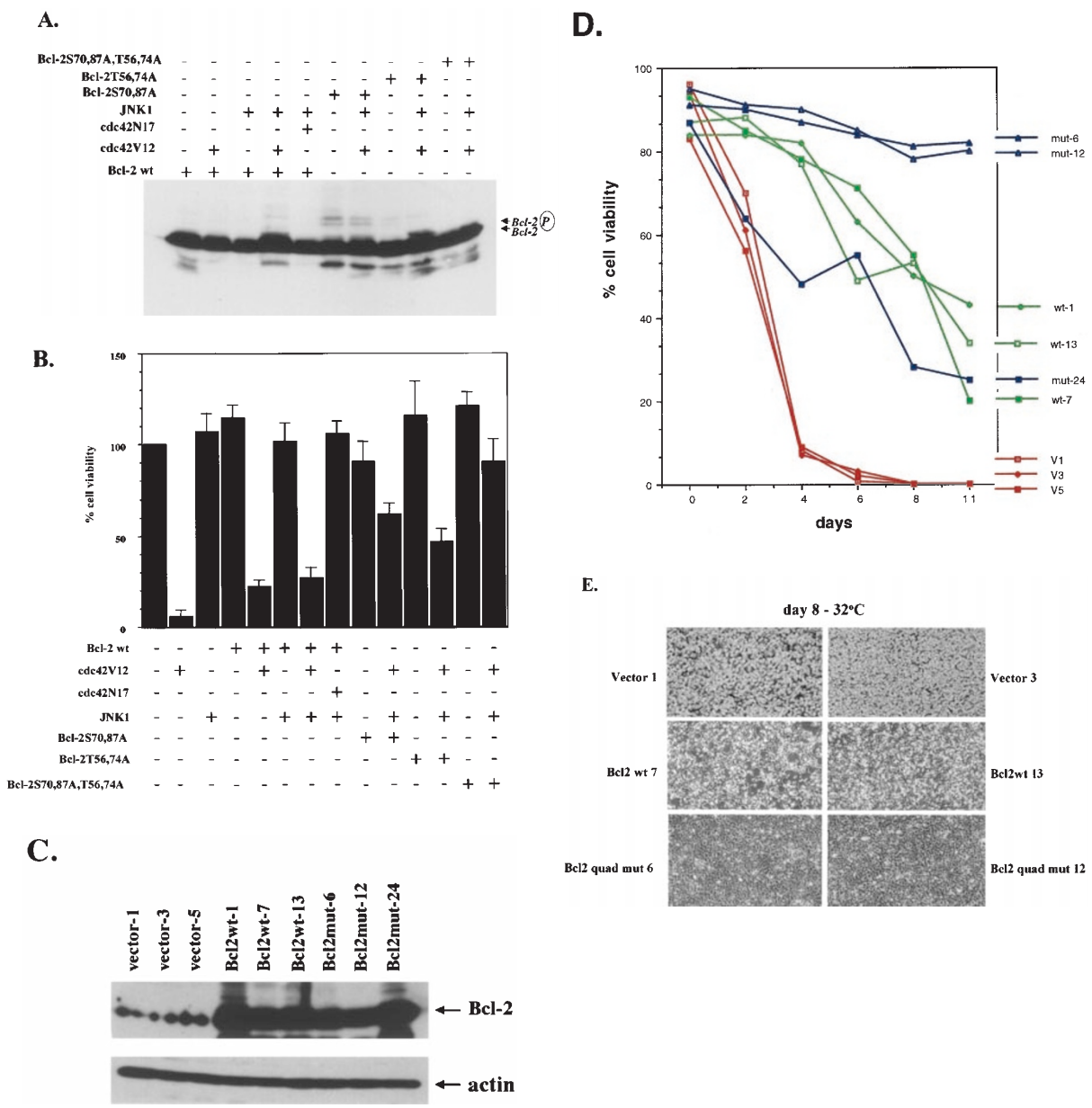

Figure 4 Cdc42-activated JNK1 phosphorylates Bcl-2 and inactivates its anti-apoptotic function. (**a**) Western blot analysis of Bcl-2 in p53A cells cotransfected with pCGcdc42V12, pcGcdc42N17, pcDNA3JNK1, pcDNA3.1Bcl-2wt, pcDNA3.1Bcl-2S70,87A, pcDNA3.1Bcl-2T56,74A, pcDNA3.1Bcl-2S70,87A,T56,74A. (**b**) Cell viability was determined by staining for β-galactosidase activity in p53A cells cotransfected with all of the plasmids mentioned in (**a**) along with 3.3 μg of pcDNA3.1lacZ. (**c**) Western blot analysis of Bcl-2 in stable clones of p53A cells expressing vector alone, Bcl-2wt, and Bcl-2S70,87A,T56,74A quadruple mutant. (**d**) Cell viability measured by trypan blue exclusion in stable clones expressing vector alone (red), Bcl-2wt (green), and Bcl-2S70,87AT56,74A (blue) quadruple mutant incubated at 38.5°C (day 0) and at 32°C from day 1 to day 11. (**e**) Analysis of stable clones expressing Bcl-2wt and Bcl-2S70,87A,T56,74A quadruple mutant incubated for 8 days at 32°C by light microscopy at a magnification of 200× to detect apoptotic cells

Inactivation of Bcl-2 by Cdc42-activated JNK1 phosphorylation

Previous studies have shown that Rac1 and Cdc42 GTPases can efficiently stimulate the c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs) (Bagrodia *et al*., 1995; Coso *et al*., 1995; Minden *et al*., 1995). Activation of these kinases can stimulate phosphorylation of transcriptional regulators such as c-Jun, ATF2, ELK and p53 (Fuchs *et al*., 1998; Gille *et al*., 1995; Gupta *et al*., 1995; Hu *et al*., 1997; Minden *et al*., 1995). Studies have shown that the activated small GTPase Rac1 can induce phosphorylation of Bcl-2 through the JNK1 kinase pathway (Maundrell *et al*., 1997). Furthermore, studies have suggested that phosphorylation of Bcl-2 inactivates its anti-apoptotic activity (Haldar *et al*., 1995). Our data have shown that Bcl-2 was ineffective at blocking Cdc42-mediated apoptosis at 38.5°C (Figure 3a). Therefore, we wanted to investigate whether Cdc42 could inactivate Bcl-2 by inducing phosphorylation through JNK1. Overexpression of activated Cdc42V12 mutant at 38.5°C did not induce a mobility shift in Bcl-2, indicative of phosphorylation (Maundrell *et al*., 1997) on SDS–PAGE (Figure 4a). However, coexpression of Cdc42V12 and JNK1 at 38.5°C induced a mobility shift of Bcl-2 on SDS–PAGE (Figure 4a). These results may indicate post-translational phos-

phorylation of Bcl-2 (Maundrell *et al*., 1997). In contrast to the activated Cdc42V12 mutant, overexpression of dominant-negative Cdc42N17 mutant along with JNK1 at 38.5°C did not induce mobility shift of wild-type Bcl-2 (Figure 4a). Furthermore, coexpression of Cdc42V12 mutant with Bcl-X_L did not induce a mobility shift of Bcl-X_L on SDS–PAGE (data not shown). In order to determine whether the mobility shift was indeed Bcl-2 phosphorylation, we mutated all the known phosphorylation sites of Bcl-2 (Maundrell *et al*., 1997). Serines 70, 87 and/or threonines 56, 74 of Bcl-2 were substituted for alanines, rendering Bcl-2 resistant to phosphorylation (Maundrell *et al*., 1997). Mobility shift of Bcl-2 double mutants S70,87A and T56,74A, was still observed, when expressed with Cdc42V12 and JNK1 (Figure 4a). However, mobility shift of Bcl-2 quadruple mutant, S70,87A,T56,74A, was consistently decreased (Figure 4a), indicating that the mobility shift may indeed represent phosphorylation of Bcl-2. Our functional studies of Bcl-2 indicated that the degree of phosphorylation correlated well with Bcl-2's ability to block Cdc42-induced cell death. Bcl-2 T56,74A mutant, which retained its ability to be phosphorylated, was not able to inhibit Cdc42-induced cell death (Figure 4b). However, the Bcl-2 S70,87A mutant, which was decreased in its phosphorylation, was a better inhibitor of Cdc42-induced cell death than wild-type Bcl-2. The Bcl-2, S70,87A,T56,74A quadruple mutant, which was resistant to phosphorylation, was able to efficiently block Cdc42-induced apoptosis (Figure 4b). The Bcl-2 mutants that are unable to be phosphorylated can block Cdc42-induced apoptosis more efficiently than wild-type Bcl-2. These results suggested that Cdc42-activated JNK1 phosphorylation of Bcl-2 inactivated its anti-apoptotic function, thereby promoting cell death.

Previous studies have demonstrated that Bcl-2 gene expression is repressed in the presence of wild-type p53 (Miyashita *et al*., 1994a,b). We have found that Bcl-2 mRNA is slightly repressed in the presence of wild-type p53 using oligonucleotide microarray technology (Cuconati and White, unpublished observations). Unfortunately, we were not able to detect Bcl-2 protein in p53A at 32°C due to the unavailability of a rat-specific Bcl-2 antibody. Nonetheless, we have previously observed that either stable expression of Bcl-2 or E1B 19K can block p53-dependent apoptosis (Chiou *et al*., 1994; Han *et al*., 1996). However, unlike E1B 19K, Bcl-2 protection is not long-lasting (Sabbatini *et al*., 1995a). The protection observed by Bcl-2 diminishes as p53-dependent cell death progresses. In contrast, E1B 19K protects cells from p53-dependent apoptosis as long as the death stimuli persist, perhaps because 19K function has not been found to be regulated by phosphorylation (McGlade *et al*., 1989). To examine more closely the role of Bcl-2 phosphorylation in p53-mediated cell death, we made stable p53A cells expressing wild-type Bcl-2 or the quadruple mutant of Bcl-2 (S70, 87A, T56, 74A). All the stable cell lines express comparable levels of the exogenous Bcl-2 protein (Figure 4c). Cell viability of the stable lines was assessed at the permissive temperature. Cells expressing both forms of Bcl-2 were resistant to the initial stages of p53-dependent apoptosis. However, viability was decreased in all three clones expressing wild-type Bcl-2 after prolonged incubation at 32°C (Figure 4d,e). In contrast, two out of three clones expressing Bcl-2S70,87A,T56,74A quadruple mutants were remarkably resistant to prolonged incubation at the permissive temperature (Figure 4d,e). These results suggest that Bcl-2 may be inactivated during p53-dependent apoptosis by being phosphorylated, possibly by JNK1. Unfortunately, the lack of a good antibody that recognizes rat Bcl-2 prevents us from determining the status of endogenous Bcl-2 during p53-mediated apoptosis in p53A cells. Nevertheless, our model suggests a mechanism whereby Bcl-2 inactivation promotes p53-dependent cell death.

Discussion

The tumor suppressor p53 is known to transcriptionally regulate several genes involved in cell cycle arrest and apoptosis (Ko and Prives, 1996; Levine, 1997). In this study, we have shown that p53 not only up-regulates Bax, it can indirectly regulate Cdc42 GTPase. Although *cdc42* transcript was only marginally up-regulated by p53, Cdc42 protein was substantially induced in the presence of wild-type p53 (Figure 5). Our results demonstrate that Cdc42 was found in its GTP-bound active form, even in the presence of mutant p53. However, cytosolic Cdc42 was translocated to the plasma membrane when wild-type p53 was activated (Figure 5). Therefore, we propose that increased protein levels and translocation of Cdc42 to the plasma membrane are probably more crucial in propagating the apoptotic signal induced by p53.

The small G proteins have been shown to induce apoptosis in lymphocytes (Gulbins *et al*., 1996) as well as in neuronal cells (Bazenet *et al*., 1998). In our system, Cdc42 GTPase was a potent inducer of apoptosis. The morphological changes during apoptosis were observed within 12 h after overexpression of activated Cdc42 mutant. Since a prominent role of Cdc42 is to effect changes in the cytoskeletal structures within cells (Hall, 1998), we cannot exclude the possibility that Cdc42 may induce cytoskeletal changes in dying cells, which probably contributes to its potency as pro-apoptotic regulator. A dominant-negative mutant of Cdc42 was not able to completely block p53-mediated apoptosis, probably due to other pathways being utilized by p53 to induce cell death. The pro-apoptotic Bax protein is directly up-regulated

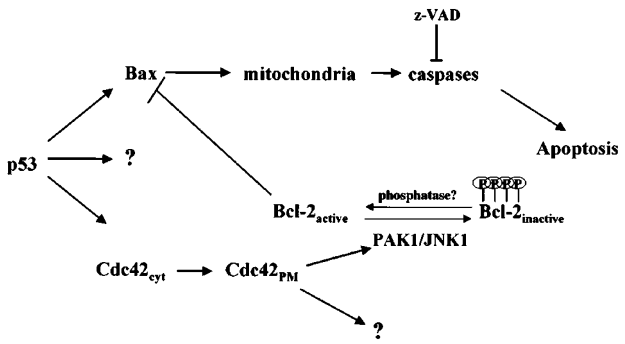

Figure 5 A model illustrating various pathways of p53-dependent cell death (details in the text)

by p53 (Miyashita and Reed, 1995). Induction of Bax promotes cytochrome *c* release from the mitochondria, which is inhibited by anti-apoptotic Bcl-2 family members (Rossé *et al*., 1998) (Figure 5). Bax facilitates the release of cytochrome *c* from the mitochondria, thereby initiating activation of caspases during p53-dependent cell death (Figure 5). However, Bax induction is not sufficient for apoptosis by p53 (McCurrach *et al*., 1997; Sakamuro *et al*., 1997). Therefore, blocking one pathway would not be sufficient for complete inhibition of cell death (Figure 5). Furthermore, previous studies have shown that caspase inhibitor, z-VAD, inhibits p53-mediated apoptosis (Sabbatini *et al*., 1997), thereby suggesting that caspase activation lies downstream of p53 (Figure 5). Our data concerning p53 induction of Cdc42 is consistent with the observation that z-VAD blocks p53-dependent cell death.

Our data demonstrated that PAK1 might be activated downstream of Cdc42 (Figure 5). However, p53-dependent apoptosis was not completely inhibited by dominant-negative PAK1. These results indicate that: (1) there are other pathways for p53 to induce apoptosis and (2) there may be other substrates utilized by Cdc42 to induce apoptosis. Reports have shown that PAK1 is activated by caspase-3 during apoptosis (Walter *et al*., 1998). Furthermore, caspase-3 is known to be activated during p53-mediated apoptosis (Sabbatini *et al*., 1997). In light of this evidence, it is possible that PAK1 activation will be an indirect consequence of apoptosis, and not direct activation by Cdc42. PAKs are well known substrates of Cdc42. However, some proteins are more specific for Cdc42 such as the activated Cdc42-associated tyrosine kinases (ACK) (Manser *et al*., 1993; Yang and Cerione, 1997) and the Wiscott-Aldrich-syndrome proteins (WASP) (Aspenstrom *et al*., 1996; Symons *et al*., 1996). We cannot rule out the possibility that these proteins may also be part of the pathway whereby Cdc42 induces cell death (Figure 5).

Other small G proteins such as Rac 1 and Rho may play a role in p53-mediated apoptosis. In our hands, an activated Rac 1 mutant was not as efficient as the activated Cdc42 mutant in inducing apoptosis (data not shown). However, it has been shown that activated Rac 1 can induce JNK1 phosphorylation of Bcl-2 (Maundrell *et al*., 1977). In addition, Rac 1 has been shown to mediate Fas-induced apoptosis in lymphocytes (Gulbins *et al*., 1996). It remains possible that other small G proteins will also play a role in p53-dependent apoptosis. Recent reports have identified small GTPases and factors associated with this family of proteins, such as rhoHP1, Cdc42Hs kinase Ack, and Ral, a GDP dissociation stimulator, as p53-regulated gene products (Zhao *et al*., 2000). This study further supports our data that the small G protein Cdc42 plays a role in the p53 pathway.

Phosphorylation of Bcl-2 has been shown to inactivate (Haldar *et al*., 1995) and activate (May *et al*., 1994) the anti-apoptotic activity of Bcl-2. Since Bcl-2 did not protect cells from Cdc42-mediated apoptosis, whereas Bcl-X_L and E1B 19K did, we investigated whether phosphorylation of Bcl-2 can inactivate it in the Cdc42 pathway. Four putative Ser/Thr kinase phosphorylation sites (Ser70, Ser87, Thr56, Thr74) in the N-terminal loop region of Bcl-2 have been reported (Maundrell *et al*., 1997). The number of sites phosphorylated by JNK1 probably depends on the intensity of kinase activation. Our data demonstrate that mutating all four sites to alanine decreased Bcl-2 phosphorylation. Although with poor resolution, we have observed metabolic ^{32}P-labeling of Bcl-2 in p53A cells expressing wild-type Bcl-2, but not Bcl-2 quadruple mutant, when cotransfected with Cdc42V12 and JNK1 (data not shown). The phosphorylated form of Bcl-2 may less efficiently interact with the proapoptotic protein Bax, therefore, Bax may be free of any antagonistic stimuli (Figure 5). Our data using the Bcl-2 quadruple mutant stable cell lines indicate that Bcl-2 phosphorylation is important for inactivating its antiapoptotic activity. We did, however, observe that one Bcl-2 quadruple mutant cell line (Bcl-2 mut-24) was sensitive to p53-dependent apoptosis, which may be due to genomic instability. For this reason, we have examined several Bcl-2 quadruple mutant stable lines and for the most part, they are truly resistant to p53-dependent apoptosis. However, it is important to emphasize that Cdc42 is not the only target for p53 and Bcl-2 may not be the only target of Cdc42 (Figure 5). Furthermore, other kinases including Raf (Blagosklonny *et al*., 1996), PKCα (Ruvolo *et al*., 1998), ERK (Deng *et al*., 2000), and Cdc2 (Furukawa *et al*., 2000) have been shown to phosphorylate Bcl-2. We have not examined the role of these kinases in Cdc42-mediated apoptosis. Therefore we cannot exclude the possibility that one or more of these kinase may play a role in phosphorylation of Bcl-2 during p53-dependent apoptosis.

Threonine 69 in the loop region of Bcl-2 has also been shown a substrate for JNK1 (Yamamoto *et al*., 1999). This is likely because our data when using the quadruple mutant of Bcl-2 occasionally displayed a slight mobility shift on SDS–PAGE, suggesting the existence of other phosphorylation site(s). Therefore, it is possible that threonine 69 may be another target site for JNK1. Recent reports have shown that Bcl-2 is phosphorylated during the G2/M phase of the cell cycle (Yamamoto *et al*., 1999). Serine 70 was observed to be the major phosphorylation site during G2/M (Yamamoto *et al*., 1999). The many phosphorylation sites of Bcl-2 may be differentially regulated by more than one kinase for different cellular processes. ASK1 and MKK7 have been shown to activate JNK1 leading to Bcl-2 phosphorylation (Yamamoto *et al*., 1999). It is likely that these kinases may be involved in JNK1 activation in our system. However, our data using dominant-negative Cdc42 show a complete inhibition of Bcl-2 phosphorylation, which strongly suggests a role for Cdc42 in the activation of JNK1 (Figure 5). In addition, recent reports have shown that JNK kinases are crucial in inducing apoptosis during DNA damage (Tournier *et al*., 2000). Primary murine embryo fibroblasts with targeted disruptions of JNK kinases were resistant to DNA damage induced by UV radiation. These reports support the idea that JNK kinases play a role in p53-dependent apoptotic pathway. Finally, JNK1 has been shown to phosphorylate p53, thereby increasing its transcriptional activity (Fuchs *et al*., 1998; Hu *et al*., 1997). This study raises the possibility that other substrates may exist for JNK1 in our system. An intriguing

hypothesis is that phosphorylation of p53 by JNK1 is a positive feedback loop whereby p53's ability to transcribe genes required for apoptosis is enhanced.

Materials and methods

Cell lines and culture conditions

Primary Fisher baby rat kidney (BRK) epithelial cells prepared from 6 day old baby rats were cultured in Dulbecco's modified eagle medium (DMEM, Life Technologies, Grand Island, NY, USA) with 5% fetal calf serum (Gemini BioProducts, Woodland, CA, USA) as described previously (White *et al*., 1992). The transformed BRK cell line, p53A, was derived from transfection of primary BRK cells with plasmids encoding E1A (pCMVE1A) and the temperature sensitive mutant p53(val135) (pLTRcGval135) (Debbas and White, 1993). The temperature sensitive p53 mutant is in the mutant conformation at the nonpermissive temperature of 38.5°C and is in the wild-type conformation at the permissive temperature of 32°C. The 22/23p53(val135) cell line was generated from primary BRK cells transformed with adenovirus E1A and p53(22–23val135) (Sabbatini *et al*., 1995b). p53(22–23val135) was generated from p53(val135) by mutating amino acids 22 and 23 which renders the protein transcriptional defective and apoptosis defective at the permissive temperature (Sabbatini *et al*., 1995b).

PCR-select cDNA subtraction

cDNA was synthesized from 1 μg of poly(A)$^+$ RNA from p53A cells at the nonpermissive temperature (38.5°C) and 4 h at the permissive temperature (32°C). The cDNA at the nonpermissive temperature was used to subtract from cDNA at the permissive temperature through several steps of hybridization and PCR amplification according to manufacturer's instructions (ClonTech, Palo Alto, CA, USA). Thus, the subtracted cDNA library enriches for p53-inducible genes, which are up-regulated after 4 h of incubation at the permissive temperature.

Plasmids

pRK5cdc42wt, pRK5cdc42L61, and pRK5cdc42N17 expression plasmids that encode for wild-type Cdc42, constitutively activated form of Cdc42 and dominant-negative form of Cdc42, respectively, were generous gifts from Dr A Hall (MRC, London, UK). Dr L Van Aelst (Cold Spring Harbor Laboratories, NY, USA) provided pCGcdc42V12 expression plasmid, which encodes for a constitutively activated form of Cdc42. pcDNA3.1cdc42N17 used for stable transfection was constructed by subcloning pRK5cdc42N17 into pcDNA3.1 using *Bam*H1 and *Eco*R1 sites. pcDNA3E1B19K, pCMVE1A and pLTRcGval135 were previously described (Debbas and White, 1993; Sabbatini *et al*., 1995a,b). Dr C Thompson (University of Pennsylvania, Philadelphia, PA, USA) provided pcDNA3Flag-Bcl-x_L, and Dr JS Gutkind (NIH, Bethesda, MD, USA) provided pcDNA3HA-JNK1. Dr C Gelinas (CABM/UMDNJ, Piscataway, NJ, USA) provided pcDNA3.1Bcl-2wt. pcDNA3.1HisLacz was purchased from Invitrogen (Carlsbad, CA, USA). pcDNA3-PAK1 and pcDNA3dominant-negative PAK1, pcDNA3.1Bcl-2S70,87A, pcDN3.1Bcl-2T56,74A, and pcDNA3.1Bcl-2S70,87A,T56,74A were constructed as described below.

Northern blot analysis

Cytoplasmic RNA was extracted from p53A cells using the NP-40 lysis protocol as previously described (Muraoka *et al*., 1996). Cytoplasmic RNA (30 μg) was loaded onto a formaldehyde gel and blotted overnight using Hybond-N membranes (Amersham Pharmacia BioTech, Piscataway, NJ, USA) for transfer. The membrane was hybridized with a random-primed α-^{32}P-dATP labeled *cdc42*, *p21^{WAF1}* or *actin* cDNA probe in ExpressHyb Hybridization solution (ClonTech, Palo Alto, CA, USA) at 65°C overnight. Blots were washed in 0.1 × SSC and 0.1% SDS at 50°C and exposed to film overnight.

Mutagenesis of PAK1 and Bcl-2

PAK1 was PCR cloned from a HeLa cDNA library. The plasmid was constructed by 3-fragment ligation of pcDNA3 (cut with *Kpn*I and *Eco*RI), FLAG-tag fragment (*Kpn*I and *Xma*I), and PAK1 PCR fragment (cut with *Xma*I and *Eco*RI). The dominant-negative form of PAK1 was constructed by substituting the active lysine 299 in the kinase domain for an arginine by PCR mutagenesis using the Stratagene (La Jolla, CA, USA) QuikChange Site-directed Mutagenesis Kit according to manufacturer's recommendations. The sense primer used was 5′-AGGAGGTGGCCATT-CGGCAGATGAATCTTCA-3′ and the antisense strand was 5′-TGAA GATT CAT CTG CCGA AT GG CC ACC TCCT-3′ (Tang *et al*., 1997).

The Bcl-2 phosphorylation mutants were constructed using the following strategy: a 5′ primer along with a mutagenic primer was used to PCR amplify parts of Bcl-2 with the specific mutation. The PCR product was run on a 1% agarose gel and purified using the Qiagen DNA purification kit (QIAEX II, Qiagen, Valencia, CA, USA) according to manufacturer's recommendations. This PCR product containing the desired mutation was then used as a 5′ primer along with a 3′ primer to amplify the entire Bcl-2 cDNA with the respective mutations. This 878 bp mutated Bcl-2 PCR product was gel purified and ligated into the TA cloning vector pcDNA3.1-V5/His (Invitrogen, Carlsbad, CA, USA). For the single mutations, wild-type Bcl-2 in pcDNA3.1 was used as the DNA template in PCR. For the double mutant Bcl-2S70,87A, the DNA template was pcDNA3.1V5/HisBcl-2S70A. The triple mutant T56A,S70,87A was made by using pcDNA3.1V5.HisBcl-2S70,87A, as the DNA template in PCR with mutagenic primer T56A. This triple mutant was then used as the DNA template in PCR to generate quadruple mutant T56,74AS70,87A with the mutagenic primer S70A,T74A.

The Bcl-2 5′ sense primer used with a *Hind*III site was 5′-GAGAAGCTTGTGCCGTTGGCCCCC-3′ and the 3′ antisense primer with an *Xba*I site used was 5′-GGTTCTAGAA-CAGCCTGCAGCTTT-3′. The following primers were used for mutagenesis: for S70A, 5′-GGTCGCCAGGACCGCGC-CACTACAGACCC-3′, for S87A, 5′-CCTGCGCTCGCCCC-GGTGCCACC-3′ , for T56A, 5′GCCCGGGCACGCGCCC-CATCCAGC-3′, for T74A, 5′-CCTCGCCACTACAGGCC-CCGGCTGC-3′, and for S70A and T74A: 5′-CCGCGCCA-CTACAGGCCCCGGCTGC-3′.

Stable and transient transfection

For stable transfections, p53A cells were electroporated (220 V, 950 μF) with 1 μg of pcDNA3.1, pcDNA3.1cdc42N17, pcDNA3dominant-negativePAK1, pcDNA3.1Bcl-2, or pcDNA3.1Bcl-2S70,87A,T56,74A, along with 10 μg of salmon sperm DNA. Cells were selected 48 h post-transfection in DMEM containing 0.5 mg/ml G418 (Life Technologies, Grand Island, NY, USA) for 14 days. Individual colonies were cloned using cloning cylinders and expanded into cell lines. For transient transfections, cells were electroporated with 8 or 10 μg of plasmid DNA and 2.7 or 3.3 μg, respectively, of pcDNA3.1HisLacZ. Cells were assayed for viability 24 h post-transfection as described below and whole cell lysates were made for monitoring protein expression. For Cdc42 transient transfections, 8 μg of

pRK5cdc42wt, pRK5cdc42L61, pCGcdc42V12, and pRK5 cdc42N17 were electroporated into p53A cells. Ten μg of pcDNA3E1B19K, pcDNA3Flag-Bcl-x_L, pcDNA3HA-JNK1, pcDNA3.1Bcl-2wt, pcDNA3.1Bcl-2S70,87A, pcDN3.1Bcl-2T56,74A, and pcDNA3.1Bcl-2S70,87A,T56,74A were electroporated into p53A cells.

Cell viability assays

Cell viability was measured by trypan blue (Life Technologies, Grand Island, NY, USA) staining. Cell were trypsinized, centrifuged, resuspended in PBS, and counted using a hemocytometer after diluting in trypan blue. β-galactosidase staining was also performed to determine cell viability in cells cotransfected with pcDNA3.1HisLacZ. Cells were fixed in 1% glutaraldehyde (Sigma, St. Louis, MO, USA) for 15 min at room temperature, and stained with X-gal (Roche Diagnostics, Indianapolis, IN, USA) for β-galactosidase activity. Blue cells were counted as an indicator of viable cells.

Indirect immunofluorescence

Cells were fixed in -10°C methanol for 10 min and washed in PBS before incubation with primary antibodies. After a few washes in PBS, cells were incubated with rhodamine-conjugated goat anti-rabbit antibody (Jackson Labs, Bar Harbor, ME, USA). Cells were then washed, mounted, and analysed by fluorescence microscopy using Nikon FXA epifluorescence microscope (Micron Optics, Parsipanny, NJ, USA).

Western blot analysis

Cell lysates were made by lysing in 2× Laemmli buffer containing 4% SDS and 5% β-mercaptoethanol. Equal amounts of protein (20 or 30 μg) were electrophoresed on 15 or 17% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked for 15 min at room temperature in 5% nonfat Carnation dry milk in PBS containing 0.1% Tween-20 (PBST). Membranes were incubated with primary antibody followed by washes in PBST and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 1 : 2000 dilution. After several washes, the blots were developed using the ECL chemiluminescence detection kit according to manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Affinity precipitation

PAK1 binding domain (residues 67–150) was immobilized on agarose beads and used as an affinity ligand for GTP-bound Cdc42 according to manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). Briefly, p53A cells were incubated at 38.5°C (day 0) and at 32°C from day 1 to day 4 and were lysed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol in the presence of 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM sodium fluoride and 1 mM sodium orthovanadate (Benard *et al*., 1999). The insoluble material was removed by centrifugation. The supernatant represents the soluble fraction, which was used for the affinity precipitation, and the pellet represents the insoluble fraction. The soluble fraction was incubated with PAK1 agarose beads for 60 min at 4°C. The beads were washed several times and resuspended in 2× Laemmli sample buffer and electrophoresed on a 17% SDS–PAGE. Western blot analysis of Cdc42 was performed as mentioned above.

Antibodies

An anti-human Bcl-2 hamster antibody (BD Pharmigen, San Diego, CA, USA) was used at 1 : 1000 dilution to detect the transfected human Bcl-2 on Western blots. A polyclonal anti-Cdc42 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1 : 200 dilution for Westerns and at 1 : 50 dilution for indirect immunofluorescence to detect rat Cdc42 protein and a mouse monoclonal anti-actin antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used at 1 : 1000 dilution to detect actin on Western blots.

Acknowledgments
We would like to thank Drs Alan Hall and Linda Van Aelst for *cdc42* expression plasmids, Dr J Silvio Gutkind for JNK1 expression plasmid, Dr Celine Gelinas for Bcl-2 expression plasmid, and Dr Craig Thompson for Bcl-X_L expression plasmid. Many thanks to Dr Andy Cuconati for making the dominant-negative PAK1 expression plasmid and all the other members of the White lab for thought-provoking discussions. This work was supported by NIH grant CA60088 and the Howard Hughes Medical Institute.

References

Aspenstrom P, Lindberg U and Hall A. (1996). *Curr. Biol.,* **6,** 70–75.
Bagrodia S, Dérijard B, Davis RJ and Cerione RA. (1995). *J. Biol. Chem.,* **270,** 27995–27998.
Bazenet CE, Mota MA and Rubin LL. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 3984–3989.
Benard V, Bohl BP and Bukoch GM. (1999). *J. Biol. Chem.,* **274,** 13198–13204.
Blagosklonny MV, Schulte T, Nguyen P, Trepel J and Neckers LM. (1996). *Cancer Res.,* **56,** 1851–1854.
Boivin D and Beliveau R. (1995). *Am. J. Physiol.,* **269,** F180–F189.
Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR and Kley N. 1995). *Nature,* **377,** 646–649.
Burbelo PD, Drechsel D and Hall A. (1995). *J. Biol. Chem.,* **270,** 29071–29074.
Chiou S-K, Rao L and White E. (1994). *Mol. Cell. Biol.,* **14,** 2556–2563.
Chou JJ, Li H, Salvesen GS, Yuan J and Wagner G. (1999). *Cell,* **96,** 615–624.
Chuang TH, Hahn KM, Lee JD, Danley DE and Bukoch GM. (1997). *Mol. Biol. Cell,* **8,** 1687–1698.
Coso OA, Chiariello M, Yu J-L, Teramoto H, Crespo P, Xu N, Miki T and Gutkind JS. (1995). *Cell,* **81,** 1137–1146.
Debbas M and White E. (1993). *Genes Dev.,* **7,** 546–554.
Deng X, Ruvolo P, Carr B and May JWS (2000). *PNAS,* **97,** 1578–1583.
Dharmawardhane S, Sanders LC, Martin SS, Daniels RH and Bukoch GM. (1997). *J. Cell. Biol.,* **138,** 1265–1278.
El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer E, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.
Fuchs SY, Fried VA and Ronai Z. (1998). *Oncogene,* **17,** 1483–1490.
Furukawa Y, Iwase S, Kikuchi J, Terui Y, Nakamura M, Yamada H, Kano Y and Matsuda M. (2000). *J. Biol. Chem.,* **275,** 21661–21667.

Gille H, Strahl T and Shaw P. (1995). *Curr. Biol.,* **5,** 1191–1200.
Gross A, McDonnell JM and Korsmeyer SJ. (1999). *Genes Dev.,* **13,** 1899–1911.
Gulbins E, Coggeshall KM, Brenner B, Schlottmann K, Linderkamp O and Lang F. (1996). *J. Biol. Chem.,* **271,** 26389–26394.
Gupta S, Campbell D, Derijard B and Davis R. (1995). *Science,* **267,** 389–392.
Haldar S, Jena N and Croce C. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4507–4511.
Hall A. (1998). *Science,* **279,** 509–514.
Han J, Sabbatini P, Perez D, Rao L, Modha D and White E. (1996). *Genes Dev.,* **10,** 461–477.
Hu MC-T, Qiu WR and Wang Y-P. (1997). *Oncogene,* **15,** 2277–2287.
Johnson DI. (1999). *Microbiol. Mol. Biol. Rev.,* **63,** 54–105.
Ko LJ and Prives C. (1996). *Genes Dev.,* **10,** 1054–1072.
Korsmeyer SJ. (1995). *Trends Genet,* **11,** 101–105.
Levine AJ. (1997). *Cell,* **88,** 323–331.
Manser E, Leung T, Salihuddin H, Tan L and Lim L. (1993). *Nature,* **363,** 364–367.
Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC and Arkinstall S. (1997). *J. Biol. Chem.,* **272,** 25238–25242.
May WS, Tyler PG, Ito T, Armstrong DK, Qatsha KA and Davidson NE. (1994). *J. Biol. Chem.,* **269,** 26865–26870.
McCurrach ME, Connor TMF, Knudson CM, Korsmeyer SJ and Lowe SW. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 2345–2349.
McDonnell JM, Fushman D, Milliman CL, Korsmeyer SJ and Cowburn D. (1999). *Cell,* **96,** 625–634.
McGlade CJ, Tremblay ML and Branton PE. (1989). *Virology,* **168,** 119–127.
Minden A, Lin A, Clarel F-X, Abo A and Karin M. (1995). *Cell,* **81,** 1147–1157.
Minn AJ, Vélez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M and Thompson CB. (1997). *Nature,* **385,** 353–357.
Miyashita T, Harigai M, Hanada M and Reed JC. (1994a). *Cancer Res.,* **54,** 3131–3135.
Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994b). *Oncogene,* **9,** 1799–1805.
Miyashita T and Reed JC. (1995). *Cell,* **80,** 293–299.
Muraoka M, Konishi M, Yanoshita R, Tanaka K, Shitara N, Chong JM, Iwama T and Miyaki M. (1996). *Oncogene,* **12,** 1565–1569.
Nagata S. (1997). *Cell,* **88,** 355–365.
Oltvai ZN, Millman CL and Korsmeyer SJ. (1993). *Cell,* **74,** 609–619.
Owen-Schaub L, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang W-W, Kruzel E and Radinsky R. (1995). *Mol. Cell. Biol.,* **15,** 3032–3040.
Rossé T, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B and Borner C. (1998). *Nature,* **391,** 496–499.
Ruvolo PP, Deng X, Carr BK and May WS. (1998). *J. Biolog. Chem.,* **273,** 25436–25442.
Sabbatini P, Chiou S-K, Rao L and White E. (1995a). *Mol. Cell. Biol.,* **15,** 1060–1070.
Sabbatini P, Ham JH, Chiou S-K, Nicholson D and White E. (1997). *Cell Growth Differ.,* **8,** 643–653.
Sabbatini P, Lin J, Levine AJ and White E. (1995b). *Genes Dev.,* **9,** 2184–2192.
Sakamuro D, Sabbatini P, White E and Prendergast GC. (1997). *Oncogene,* **15,** 887–898.
Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB and Fesik SW. (1997). *Science,* **275,** 983–986.
Schendel SL, Azimov R, Pawlowski K, Godzik A, Kagan BL and Reed JC. (1999). *J. Biol. Chem.,* **274,** 21932–21936.
Sells MA and Chernoff J. (1997). *Trends Cell Biol.,* **7,** 162–167.
Symons M, Derry JMJ, Karlak B, Jiang S, Lemahieu V, McCormick F, Francke U and Abo A. (1996). *Cell,* **84,** 723–734.
Tang Y, Chen Z, Ambrose D, Liu J, Gibbs JB, Chernoff J and Field J. (1997). *Mol. Cell. Biol.,* **17,** 4454–4464.
Tapon N and Hall A. (1997). *Curr. Opin. Cell Biol.,* **9,** 86–92.
Thomas A and White E. (1998). *Genes Dev.,* **12,** 1975–1985.
Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA and Davis RJ. (2000). *Science,* **288,** 870–873.
Van Aelst L and D'Souza-Schorey C. (1997). *Genes Dev.,* **11,** 2295–2322.
Walter BN, Huang Z, Jakobi R, Tuazon PT, Alnemri ES, Litwack G and Traugh JA. (1998). *JBC,* **273,** 28733–28739.
White E. (1996). *Genes Dev.,* **10,** 1–15.
White E, Sabbatini P, Debbas M, Wold WSM, Kusher DI and Gooding L. (1992). *Mol. Cell. Biol.,* **12,** 2570–2580.
Yamamoto K, Ichijo H and Korsmeyer SJ. (1999). *Mol. Cell. Biol.,* **19,** 8469–8478.
Yang W and Cerione RA. (1997). *J. Biol. Chem.,* **222,** 24819–24824.
Zhao R, Gish K, Murphy M, Yin Y, Notterman D, Hoffman WH, Tom E, Mack DH and Levine AJ. (2000). *Genes Dev.,* **14,** 981–993.
Zhao ZS, Manser E, Chen X-Q, Chong C, Leung T and Lim L. (1998). *Mol. Cell. Biol.,* **18,** 2153–2163.