Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21^{waf1/cip1}

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Deregulated expression of the *c-myc* proto-oncogene can lead to apoptosis under certain physiological conditions. By introducing a conditionally active Myc allele into primary embryo fibroblasts null for p53, and into fibroblasts without endogenous p53 expression but ectopically expressing a temperature-sensitive p53 allele, we show that expression of wild-type p53 is required for susceptibility to Myc-mediated apoptosis. Although ectopic expression of wild-type p53 blocked cells in the G₁ phase of the cell cycle, G₁ arrest by isoleucine starvation, in a manner independent of p53, did not confer susceptibility to apoptosis. Thus, growth arrest per se is not sufficient to induce Myc-mediated apoptosis; instead, a property intrinsic to p53 is specifically required. Moreover, apoptosis did not require induction of p53 target proteins, including the cyclin-dependent kinase inhibitor p21^{waf1/cip1}. Therefore, the role of p53 in apoptosis may be distinct from its role in cell cycle arrest.

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The regulation of cell proliferation involves a delicate balance between the actions of proto-oncogenes and tumor suppressor genes. Deregulation or inactivation of either set of genes can lead to uncontrolled cell proliferation, terminal cell cycle arrest, or enhanced activation of programmed cell death (apoptosis), all of which have potentially devastating consequences to an organism. Thus, tremendous selective pressure must exist for the evolution of mechanisms to regulate these genes, at the levels of both the synthesis and activities of their gene products.

The *c-myc* proto-oncogene plays an important role in the control of proliferation and differentiation (for review, see Cole 1986; Lüscher and Eisenman 1990; Kato and Dang 1992; Marcu et al. 1992; Evan and Littlewood 1993). Expression of Myc is required and in some cases sufficient for the G₁/S phase transition (Eilers et al. 1991; Evan et al. 1992; Wagner et al. 1993b), and enforced expression of Myc can prevent cellular differentiation (Marcu et al. 1992). In addition, Myc may also play a role in the G₂/M transition (Shibuya et al. 1992; Wagner et al. 1993b). Furthermore, deregulated Myc expression has been observed in a variety of neoplasias, and experimental overexpression of Myc in cells and animals

has led to cellular transformation and oncogenesis (for review, see Cole 1986; Cory 1986; Bishop 1991; Spencer and Groudine 1991; Marcu et al. 1992).

The mechanisms by which Myc exerts such profound effects on the regulation of cell cycle progression have until recently remained obscure. Descriptions of Myc as a sequence-specific transcriptional activator (Benvenisty et al. 1992; Kretzner et al. 1992; Reddy et al. 1992; Amin et al. 1993; Reisman et al. 1993) suggest that Myc may act by directly regulating expression of proteins required for cell cycle progression, such as ornithine decarboxylase (Bello-Fernandez et al. 1993; Wagner et al. 1993a) and the G₁ cyclins (Jansen-Dürr et al. 1993 and A.J. Wagner and N. Hay, unpubl.). This possibility is strengthened by the observation that regions of the Myc protein that are required for transcriptional activation colocalize to those required for transformation (Stone et al. 1987; Kato et al. 1990; Amin et al. 1993). Myc may also suppress the expression of genes associated with growth arrest and differentiation (Freytag and Geddes 1992). In addition, Myc has been reported to interact with the tumor suppressor p107 (Gu et al. 1994), and this association may also play a role in cell cycle control.

Myc has also been paradoxically demonstrated to induce programmed cell death when expressed without appropriate growth factor stimulation or in cells arrested by other means (Askew et al. 1991; Evan et al. 1992; Shi

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et al. 1992). These opposing Myc-inducible pathways of cell cycle progression and cell death lend an extra level of complexity to tumorigenesis induced by Myc deregulation. That is, what determines to which route Myc deregulation will lead? And how do tumors arise from cells in which Myc is deregulated?

One possible mechanism is that in the context of an inhibited or blocked cell death pathway, Myc overexpression will favor proliferation and tumor formation. If this is the case, tumors with deregulated Myc expression should contain a second genetic lesion that inactivates the cell death pathway. Several models of Myc-induced tumorigenesis support this concept, because coexpression of a second oncogene or activation of a collaborator accelerates the rate of tumor formation (Haupt et al. 1991; Möröy et al. 1991; van Lohuizen et al. 1991). This mechanism can be tested experimentally by interference with the cell death pathway, either by overexpression of genes that block cell death or by disruption of genes required for cell death.

Previous work by our laboratory and others has demonstrated that overexpression of the *bcl-2* proto-oncogene can inhibit Myc-mediated apoptosis in Rat 1a fibroblasts (Fanidi et al. 1992; Wagner et al. 1993b) and in Chinese hamster ovary cells (Bissonnette et al. 1992). This observation may account for the natural history of the most common form of human follicular lymphomas, which arise as indolent tumors blocked in cell death due to overexpression of Bcl-2 but frequently progress into aggressive malignancies following Myc deregulation (McDonnell and Korsmeyer 1991; Reed 1994). That is, disruption of the cell death pathway allows cells to tolerate Myc deregulation and leads to a more rampant malignancy. This situation is likewise observed in transgenic animals harboring Bcl-2 transgenes, which also develop lymphomas with Myc deregulation (McDonnell and Korsmeyer 1991).

We have speculated previously that the p53 tumor suppressor may play a role in regulating Myc-mediated apoptosis as well (Wagner et al. 1993b). This speculation is based on the following suggestive observations: (1) p53 is frequently mutated, deleted, or inactivated in a variety of tumors and tumor cell lines that exhibit *myc* deregulation or amplification. These include the myeloid leukemia cell line HL60, which has amplification of the *c-myc* gene and deletion of *p53* (Dalla-Favera et al. 1982; Wolf and Rotter 1985); small cell lung carcinomas, which have amplification of L- or *c-myc* and *p53* mutations (Nau et al. 1985; Takahashi et al. 1990); and Burkitt lymphomas and pre-B and T cell acute lymphoblastic leukemias, which have deregulation of *c-myc* and *p53* mutations (Farrell et al. 1991; Wiman et al. 1991). (2) p53 is required for apoptosis induced by γ -irradiation and by chemotherapeutic drugs in cells sensitized by overexpression of the adenovirus E1A oncogene (Lowe et al. 1993a,b), which is similar to Myc in its ability to transform cells. (3) Exogenous expression of p53 in tumor cell lines with mutant or deleted *p53* genes can induce apoptosis (Yonisch-Rouach et al. 1991; Shaw et al. 1992; Ramqvist et al. 1993; Wang et al. 1993a). (4) Myc has

been demonstrated to *trans*-activate the p53 promoter and thus may induce expression of p53 (Reisman et al. 1993).

To evaluate the requirement of p53 in Myc-mediated apoptosis, we examined the effect of Myc overexpression in primary cells and cell lines that do not express p53. We found that ectopic Myc expression does not induce apoptosis in primary cells or cell lines not expressing p53. Furthermore, introduction of a temperature-sensitive p53 allele resulted in susceptibility to Myc-induced apoptosis only when p53 was expressed in its wild-type conformation. Although expression of wild-type p53 led to growth arrest in the absence of active Myc, induction of Myc activity overcame this effect, driving cells into the cell cycle but leading to apoptosis. However, growth arrest by other means in the absence of wild-type p53 expression did not confer susceptibility to Myc-mediated apoptosis, suggesting that an activity intrinsic to p53 is necessary. Additionally, the p53-dependent apoptosis did not require *de novo* protein synthesis, including induction of the cyclin-dependent kinase (cdk) inhibitor p21^{waf1/cip1}, which is thought to mediate p53-induced cell cycle arrest (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Dulic et al. 1994). Thus, the requirement of p53 in conferring susceptibility to Myc-mediated apoptosis may be a function distinct from its ability to induce cell cycle arrest. These data demonstrate that wild-type p53 is specifically required for cell death induced by inappropriate expression of c-Myc and suggest that inactivation of p53 in tumors with deregulated Myc expression may be one mechanism by which cells have evaded control of tumorigenesis by cell death.

Results

Myc overexpression does not induce apoptosis in serum-starved p53^{-/-} mouse embryo fibroblasts

Deregulated Myc expression in cells without adequate growth factor stimulation has been demonstrated to lead to apoptosis in several cell lines (Askew et al. 1991; Evan et al. 1992). However, tumors in which Myc expression is deregulated are often able to escape cell death. One mechanism by which these tumors arise appears to be through the deregulation of Bcl-2 expression, which in turn confers resistance to Myc-mediated apoptosis (Bissonnette et al. 1992; Fanidi et al. 1992; Wagner et al. 1993b). An alternative mechanism for escape from apoptosis may be through p53 mutation, inactivation, or deletion, all of which are common events in tumorigenesis (for review, see Hollstein et al. 1991; Levine et al. 1991). Thus, we sought to examine the functional requirement of p53 expression in Myc-mediated apoptosis. To this end, we used retroviruses to constitutively express high levels of c-Myc in fibroblasts from 14.5-day mouse embryos with homozygous inactivation of both p53 alleles (Donehower et al. 1992; Harvey et al. 1993). Early-passage p53^{-/-} mouse embryo fibroblasts (MEFs) were infected with pMV12-Myc or pMV12 retroviruses (Wagner et al. 1993b) and cells with integrated retrovi-

ruses were selected in hygromycin B. Ectopic Myc expression in $p53^{-/-}$ MEFs was verified by Northern and Western blotting (data not shown). Following serum starvation, in conditions demonstrated previously to induce cell death in Rat 1a pMV12-Myc cells (Wagner et al. 1993b), no significant cell death compared to wild-type ($p53^{+/+}$) MEFs or $p53^{-/-}$ MEFs infected with the control virus (pMV12) was observed in any of the derived cell lines tested, including a pool and three independent clones of $p53^{-/-}$ MEFs overexpressing c-Myc (Fig. 1A,C).

We also infected wild-type and $p53^{-/-}$ MEFs with retroviruses encoding a Myc-estrogen receptor fusion (MycER) protein. The MycER gene encodes a chimera of full length c-Myc and the ligand binding domain of the

estrogen receptor (ER) (Eilers et al. 1989). Although it is constitutively expressed from the retroviral long terminal repeat promoter, it is a conditional Myc allele in that it requires 17β -estradiol (E2) or other ER agonists for activity. This protein has been demonstrated previously to be active in inducing transformation, cell cycle progression, transcription, and apoptosis (Eilers et al. 1989, 1991; Evan et al. 1992; Jansen-Dürr et al. 1993; Wagner et al. 1993a). Early-passage wild-type and $p53^{-/-}$ MEFs were infected with pMV12-MycER retrovirus and were selected for hygromycin resistance in the absence of estradiol and phenol red (which has slight estrogenic activities) to prevent activation of the MycER fusion protein. This precludes selection of cells specifically resis-

Figure 1. Overexpression of c-Myc does not induce cell death in serum-starved MEFs nullizygous for p53. (A) Microscopic phenotypes of MEFs after 72 hr incubation in DMEM with 0.1% FBS. Shown are wild-type ($p53^{+/+}$) MEFs and fibroblasts from the $p53$ knockout mouse ($p53^{-/-}$) infected with control (pMV12) or Myc-expressing retroviruses. [Myc.P] Pool of Myc-expressing cells; [Myc.1, Myc.3, Myc.4] three independent clones of Myc-expressing cells. (B) (Left) Western analysis of MycER expression in MEFs from the $p53$ knockout mouse. Total cellular proteins from 2×10^5 cells were analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with a rat anti-human estrogen receptor monoclonal antibody (H222). Antibody-antigen interactions were detected by enhanced chemiluminescence (ECL). (Lane 1) Lysate from cells infected with the control virus; (lane 2) lysate from cells infected with the MycER virus, displaying the 90-kD MycER fusion protein. Migrations of molecular mass standards (in kilodaltons) are shown at left. (Right) Phenotype of serum-starved $p53^{-/-}$ MEFs expressing MycER fusion protein in the absence (-E2) or presence (+E2) of $1 \mu\text{M}$ 17β -estradiol, 72 hr after treatment. (C) Viability of cells following serum starvation. Bars represent mean viability $\pm$ s.e. of triplicate determinations. (Hatched bars) 0 hr in 0.1% FBS; (stippled bars) 72 hr in 0.1% FBS; (solid bar) 72 hr in 0.1% FBS with $1 \mu\text{M}$ 17β -estradiol (E2). (D) Phenotype of serum-starved $p53^{+/+}$ MEFs expressing MycER fusion protein in the absence (-E2) or presence (+E2) of $1 \mu\text{M}$ 17β -estradiol, 20 hr after treatment.

tant to Myc overexpression because the MycER in these cells was maintained in its inactive state. MycER expression in these cells was verified by Western analysis by use of a monoclonal antibody directed against ER (Fig. 1B). As observed with p53 knockout cells expressing constitutively active Myc, no apoptosis was observed in serum-starved p53 knockout cells in the absence (inactive Myc) or presence (active Myc) of E2 (Figs. 1B,C). Although we were repeatedly unable to establish hygromycin-resistant wild-type MEFs ectopically expressing constitutively active c-Myc, we were able to express the MycER fusion in wild type MEFs in the absence of E2. In these cells, treatment with E2 after serum starvation led to apoptosis (Fig. 1D). Thus, Myc overexpression in serum-starved primary cells not expressing p53 does not induce apoptosis, contrasting with previous observations in cells with wild-type p53, including serum-starved Rat 1a fibroblasts, rat embryo fibroblasts and WI-38 cells (normal human diploid lung fibroblasts; Evan et al. 1992; Wagner et al. 1993b; data not shown).

Restoration of wild-type p53 activity in p53^{-/-} cells results in cell cycle arrest

To further elucidate the role of wild-type p53 in Myc-mediated apoptosis, we turned to a MEF cell line that deleted large portions of both p53 alleles during immortalization and as a result expresses no detectable p53 mRNA or protein. This clonal cell line [(10.1)] (Harvey and Levine 1991) and a derivative transfected with temperature-sensitive p53 [(10.1)Val5] (Wu et al. 1993) were infected with pMV12–MycER or pMV12 retroviruses and selected in hygromycin B. Expression of p53 and MycER was verified by immunoprecipitation (not shown) and Western analysis (Fig. 2A), respectively. These resulting cell lines allow modulation of p53 activity by temperature shift and rapid induction of Myc activity by addition of E2.

The temperature-sensitive p53 allele has been demonstrated previously to be able to induce cell cycle arrest, differentiation, or apoptosis when expressed in cells at the permissive temperature (Diller et al. 1990; Michalovitz et al. 1990; Martinez et al. 1991; Yonisch-Rouach et al. 1991; Johnson et al. 1993; Wang et al. 1993a; Woodworth et al. 1993). Accordingly, we first analyzed the effect of wild-type p53 activity in the absence of overexpression of active Myc. Cell death and cell proliferation were analyzed by microscopy (Figs. 2B,C), analysis of growth rates (Fig. 3), and by flow cytometry (Fig. 4). In the absence of E2, the growth rate of (10.1) MycER cells was not significantly altered by shift to 33°C as compared to the rate at 39°C (Figs. 2B and 3A). However, (10.1)Val5 MycER cells were noticeably growth arrested at 33°C (Figs. 2C and 3B). Flow cytometric cell cycle analysis confirmed that these cells arrested in the G₁ phase of the cell cycle (Fig. 4A; Table 1). This agrees with previous reports that p53, when expressed in its wild-type conformation, can induce cell cycle arrest in these cells and others (Diller et al. 1990; Mercer et al. 1990; Michalovitz et al. 1990; Martinez et al. 1991; Wu and Levine 1994). Control cells infected with pMV12 retroviruses behaved exactly as their non-E2-treated MycER-expressing counterparts (data not shown).

Myc overexpression overcomes p53-mediated growth arrest and induces apoptosis

To evaluate the effect of Myc overexpression in cells with and without wild-type or mutant p53, (10.1) MycER and (10.1)Val5 MycER cells were incubated in medium containing 10% fetal bovine serum at 33°C or 39°C for 24 hr and were then treated with 1 μM E2 or left untreated (Fig. 2B,C). Flow cytometric analysis indicated that activation of the MycER protein enabled cells to enter and stay in the cell cycle, demonstrating that Myc activity is dominant over p53-mediated G₁ growth arrest (Fig. 4 and

Figure 2. Effects of Myc and p53 on cell phenotypes. (A) Western analysis of MycER expression in (10.1) and (10.1)Val5 cells. Extracts from 2×10^5 cells were electrophoresed in an 8% SDS–polyacrylamide gel and analyzed as in Fig. 1. (Lanes 1,3) Lysates from (10.1) and (10.1)Val5 cells infected with the control pMV12 virus; (lanes 2,4) lysates from (10.1) and (10.1)Val5 cells infected with the MycER virus, displaying the 90-kD MycER fusion protein. Migrations of molecular mass standards (in kilodaltons) are shown at left. (B) Effect of E2 and temperature shift on phenotypes of (10.1) MycER cells. Pictured are cells 48 hr after temperature shift from 37°C to 33°C or 39°C in the absence (–E2) or presence (+E2) of 1 μM E2. (C) Effect of E2 and temperature shift on phenotypes of (10.1)Val5 MycER cells. Cells were treated as described in B.

Figure 3. Growth curves following temperature shift and E2 treatment. (A) Numbers of (10.1) MycER cells after various times at 33°C (open symbols) and 39°C (filled symbols) in the absence (squares) or presence (triangles) of E2. (B) Numbers of (10.1)Val5 MycER cells after various times at 33°C (open symbols) and 39°C (filled symbols) in the absence (squares) or presence (triangles) of E2.

Table 1). However, as shown in Figure 2C, cell death was readily observable in the (10.1)Val5 MycER cells following treatment with E2 at the permissive temperature but not at the restrictive temperature. Untreated cells, as described above, were growth arrested at 33°C but not at 39°C. No growth arrest or increase in cell death was observed in the E2-treated (10.1) MycER cells at either temperature (Figs. 2B and 3A). Taken together, these data demonstrate that although ectopically expressed Myc overrides p53-induced G₁ arrest, it also leads to cell death. Induction of this pathway requires both high levels of Myc expression and the expression of wild-type p53, indicating that p53 activity is required for susceptibility to Myc-mediated apoptosis.

To verify that cell death was occurring by apoptosis, genomic DNA was prepared from equal numbers of cells

Figure 4. Flow cytometric cell cycle analysis of (10.1)Val5 MycER cells. Cells were incubated for 48 hr at in the absence (A,B) or presence (C,D) of E2 at 33°C (A,C) or 39°C (B,D), then fixed, stained with propidium iodide, and analyzed by flow cytometry. DNA content is represented by relative fluorescence. Cells in G₀/G₁ are represented by the first peak, cells in G₂/M are represented by the second peak, and cells in S are represented by the area between the peaks. Quantitation of cell cycle distributions is presented in Table 1.

that had been treated for 48 hr at 33°C or 39°C in the presence or absence of E2. Agarose gel electrophoresis of this DNA demonstrated the oligonucleosomal fragmentation pattern characteristic of apoptotic cell death only in cells expressing wild-type p53 with active Myc (Fig.

Table 1. Cell cycle distribution of (10.1)Val5 MycER cells following temperature shift and addition of E2

Conditions	Percent G ₀ /G ₁	Percent S	Percent G ₂ /M
33°C	83.4 ± 4.3	6.5 ± 1.0	10.2 ± 4.5
33°C + E2	50.4 ± 4.8	16.8 ± 6.8	32.8 ± 4.4
39°C	59.8 ± 9.6	19.3 ± 4.3	20.7 ± 6.4
39°C + E2	46.3 ± 4.5	24.7 ± 4.0	28.4 ± 6.7

Values represent means ± S.E. of triplicate measurements of cell cycle distribution determined by flow cytometry. A representative analysis is presented in Fig. 4.

5A). This was observed in both the floating cells and, to a lesser extent, the adherent cells. The apoptosis in the adherent cells was more readily detectable by a sensitive method that involves end-labeling DNA fragment termini with [^{32} P]ddATP by terminal deoxynucleotidyl transferase (Tilly and Hsueh 1993) followed by agarose gel electrophoresis (Fig. 5B). Even at longer exposures, at most minimal fragmentation was observed in other cell lines or conditions (data not shown).

Cells growth-arrested by amino acid starvation but not expressing wild-type p53 are refractory to Myc-mediated apoptosis

Because wild-type p53 expression in these cells led to growth arrest, and because other means of growth arrest in cells with normal levels of wild-type p53 resulted in susceptibility to Myc-mediated apoptosis (Evan et al. 1992), we wondered whether growth arrest was sufficient or whether p53 itself was specifically required for this process. Accordingly, we arrested (10.1)Val5 MycER cells in a manner independent of p53. As shown in Figure 6A and Table 2, cells expressing mutant p53 were arrested in G₁ by isoleucine deprivation. After treatment with E2, no significant cell death was observed even after 60 hr of starvation (Fig. 6B). However, in cells placed at 33°C, with p53 in its native conformation, or in Rat 1a cells, which express wild-type p53 (see below), the activation of MycER led to cell death within 24 hr (Fig.

6C,D). The absence of cell death in E2-treated cells at 39°C is not attributable to a gain-of-function property of mutant p53 because (10.1) MycER cells, which do not express detectable levels of p53 (Harvey and Levine 1991, and see below) were also refractory to Myc-mediated apoptosis following isoleucine starvation (data not shown). Therefore, G₁ growth arrest per se is not sufficient for susceptibility to Myc-mediated apoptosis; rather, a property intrinsic to p53 is specifically required.

p53-dependent induction of protein synthesis, including induction of p21^{waf1/cip1}, is not required for apoptosis

p53 is thought to function at least in part through its ability to regulate transcription. Its ability to cause G₁ arrest is dependent on transcriptional activation domains at the amino terminus of the protein (Pietenpol et al. 1994). p53 has been demonstrated to induce expression of a recently identified protein, p21^{waf1/cip1} (El-Deiry et al. 1993) that inhibits cdk activity and therefore is able to elicit cell cycle arrest (Harper et al. 1993; Xiong et al. 1993; Dulic et al. 1994). To address the possibility that Myc-mediated apoptosis is induced by an intrinsic property of p53 that is independent of its ability to cause growth arrest, we examined whether p53-dependent apoptosis required new protein synthesis. Apoptosis was measured by analyzing DNA fragmentation, which precedes loss of viability and allows for analysis prior to toxicity from long term cycloheximide (CHX) treatment. (10.1)Val5 MycER cells were grown at the restrictive temperature with E2 present in the media for 6 hr prior to temperature shift. CHX was added to the media 1 hr prior to temperature shift to block synthesis of p53-inducible proteins following placement of the cells at the permissive temperature. As shown in Figure 7A, DNA fragmentation was induced to similar levels in cells at the permissive temperature treated with E2 in the presence or absence of CHX. Low levels of fragmentation were observed in untreated cells or cells treated with CHX alone because of leakiness of MycER; however, DNA fragmentation was not observed in cells treated in any of the conditions at the restrictive temperature. This is also consistent with previous observations that CHX treatment does not affect Myc-mediated apoptosis in Rat 1 cells (Evan et al. 1992, and A.J. Wagner and H. Hay, unpubl.). This regimen of CHX treatment blocked induction of p21^{waf1/cip1}, otherwise detectable 4 hr after temperature shift, as shown by Western analysis (Fig. 7B).

Established cell lines expressing wild-type p53 can be resistant to Myc-mediated apoptosis

In our studies of Myc-mediated apoptosis in fibroblasts, we noticed that although Rat 1a cells, mouse and rat embryo fibroblasts, and WI-38 cells were highly susceptible, other fibroblast cell lines, including BALB/c 3T3 (A31) cells, were not (A.J. Wagner and N. Hay, unpubl.). We wondered whether this may be attributable, at least in part, to the status of p53 in these cells. Accordingly,

Figure 5. DNA fragmentation analysis. Genomic DNA was prepared from (10.1)Val5 pMV12 and (10.1)Val5 MycER cells 48 hr after temperature shift to 33°C or 39°C and treatment with E2, as indicated. (A) Ethidium bromide-stained 2% agarose gel containing DNA from approximately 4×10^5 cells per lane. Adherent (A) and floating (F) cells were separated only in the analysis of (10.1)Val5 MycER cells at 33°C with E2, as these were the only cells with significant numbers of floating cells. Migrations of restriction digested λ and ϕ X174 molecular size standards (in base pairs) are shown. (B) Agarose gel electrophoresis of 100 ng of genomic DNA prepared from cells treated as indicated. DNA fragment termini were end-labeled with [^{32} P]ddATP by terminal deoxynucleotidyl transferase prior to electrophoresis. Positions of molecular size standards are indicated.

Figure 6. Effect of amino acid starvation on susceptibility to Myc-mediated apoptosis. (A) Isoleucine starvation causes G₁ arrest in (10.1)Val5 MycER cells independently of p53. Cells were incubated at the restrictive temperature in normal DMEM (+ILE) or isoleucine-free DMEM (-ILE) for 60 hr and analyzed as described in Fig. 4. Quantitation is presented in Table 2. (B) Effect of MycER activation in isoleucine-starved (10.1)Val5 MycER cells at the restrictive temperature. Cells (10⁶) were grown at 39°C and photographed 60 hr after treatment with E2 in normal or isoleucine-free media, as indicated. Note growth to confluency in normal media but arrest without cell death in isoleucine-free media, regardless of addition of E2. (C) Cells treated as in B but at the permissive temperature (33°C). Note growth arrest in untreated cells in presence or absence of isoleucine, and prominent cell death in normal or deficient media after treatment with E2. (D) Myc-induced cell death following isoleucine starvation of cells with endogenous wild-type p53. Rat 1a MycER cells were starved of isoleucine and treated with E2 for 60 hr, as indicated.

we immunoprecipitated p53 from [³⁵S]methionine metabolically labeled cells to identify, using conformation-specific antibodies, whether indeed p53 was wild type in Rat 1a cells yet mutant in the resistant cell lines. On the basis of its immunoprecipitation with pan- and wild-type- but not mutant-specific antibodies (Harlow et al. 1981; Tan et al. 1986; Yewdell et al. 1986; Gannon et al. 1990), we found that Rat 1a cells expressed only wild-type p53 (Fig. 8). By the same criteria we observed that BALB/c 3T3 (A31) cells also expressed only wild-type

p53, as was reported previously (Gronostajski et al. 1984). In addition, we have isolated revertants of Rat 1a cells that are still transformed by Myc and express wild-type p53 yet are no longer susceptible to Myc-induced cell death (A.J. Wagner and N. Hay, unpubl.). Thus, although wild-type p53 is required for apoptosis induced by Myc deregulation, it is not sufficient, and other lesions apparently may also block the cell death pathway.

Discussion

We introduced constitutively or conditionally active alleles of c-Myc into cells that do not express p53 to evaluate the role of wild-type p53 activity in Myc-induced apoptosis. Under conditions of serum starvation, which elicits apoptosis when Myc is overexpressed in primary cells and some established cell lines with wild-type p53 (Evan et al. 1992), these cells did not undergo cell death. Introduction of a conditional p53 allele resulted in susceptibility to cell death only in conditions in which p53 was in its wild-type conformation and Myc was active. Although wild-type p53 induced cell cycle arrest in cells (in the absence of active Myc), growth arrest by p53-

Table 2. Cell cycle distribution of (10.1)Val5 MycER cells

Conditions	Percent G ₀ /G ₁	Percent S	Percent G ₂ /M
- ILE	81.7 ± 3.8	5.9 ± 2.6	12.2 ± 1.6
+ ILE	55.9 ± 4.5	22.4 ± 1.4	22.8 ± 4.8

Distribution of cells after 60 hr of isoleucine starvation (- ILE) or growth in normal media (+ ILE).

Values represent means ± s.e. of triplicate measurements of cell cycle distribution determined by flow cytometry. A representative analysis is presented in Fig. 6A.

Figure 7. Induction of apoptosis independent of p53-mediated protein synthesis. (A) Agarose gel electrophoresis of end-labeled genomic DNA. Cells [(10.1)Val5 MycER] were grown at 39°C and treated with E2 as indicated for 6 hr prior to temperature shift. One hour before temperature shift, cells, as indicated, were treated with 15 μ g/ml CHX. Cells were then shifted to 33°C or left at 39°C. Genomic DNA was prepared from cells 16 hr after temperature shift and labeled as described in Fig. 5B. (Lanes 1,5) No treatment; (lanes 2,6) E2 alone; (lanes 3,7) CHX alone; (lanes 4,8) E2 and CHX. (B) Western analysis of p21 expression following temperature shift of (10.1)Val5 MycER cells in presence or absence of 15 μ g/ml of CHX. Whole-cell lysates were prepared from cells various times after shift from 39°C to 33°C and analyzed by electrophoresis through a 12.5% SDS-polyacrylamide gel. After transfer to nitrocellulose, p21 was detected by incubation with a rabbit anti-p21 polyclonal antibody (PharMingen) followed by ECL. The positions of p21 and protein molecular size standards (in kilodaltons) are indicated. The asterisk (*) indicates a protein nonspecifically recognized by the primary antibody. (Lanes 1–6) Lysates from cells after temperature shift to 33°C for various times, as indicated; (lane 7) control lysate from cells not temperature-shifted; (lanes 8–12) lysates from cells treated with CHX beginning 1 hr prior to temperature shift.

independent means was not sufficient to make cells susceptible to Myc-mediated apoptosis. From these observations, we conclude that, at least in fibroblasts, functional p53 is required for apoptosis induced by Myc.

It is important to emphasize that although wild-type p53 is required for Myc-induced cell death, other factors

may override this pathway. Therefore, cells that express wild-type p53 may not always be susceptible to Myc-mediated apoptosis. This is demonstrated clearly in cells in which a dominant survival protein, such as Bcl-2, is expressed. For instance, Rat 1a cells, which express wild-type p53 (see above), are highly susceptible to Myc-induced apoptosis, but ectopic expression of Bcl-2, the Bcl-2-like protein Bcl-x_L [Boise et al. 1993], or adenovirus E1B 19K protein will override the induction of cell death [Fanidi et al. 1992; Wagner et al. 1993b, and A.J. Wagner and N. Hay, unpubl.]. Thus, expression of a survival gene will block apoptosis, even in the presence of wild-type p53.

Curiously, BALB/c 3T3 (A31) murine fibroblasts, which express wild-type p53 [Gronostajski et al. 1984, and see above], are not susceptible to Myc-mediated apoptosis when starved of serum growth factors [A.J. Wagner and N. Hay, unpubl.]. In this cell line, the p53-dependent apoptosis pathway has apparently been uncoupled from p53 expression, although the precise mechanism for how this occurred has yet to be determined. It is interesting to note that BALB/c 3T3 (A31) cells are exceptional in that 11 of 11 clonal fibroblast cell lines established from BALB/c embryos by a 3T3 passage schedule mutated or deleted p53 during the course of immortalization [Harvey and Levine 1991]. The genetic lesion(s) that occurred during BALB/c 3T3 (A31) immortalization may also account for its resistance to Myc-mediated apoptosis. In addition, we have isolated Rat 1a revertants that are no longer susceptible to Myc-mediated apoptosis yet still express wild-type p53 [A.J. Wagner and N. Hay, unpubl.].

Figure 8. Immunoprecipitation of p53 from various cell lines. Rat 1a, BALB/c 3T3 (A31), (10.1), and (10.1)Val5 MycER cells were labeled with [³⁵S]methionine, and p53 proteins were immunoprecipitated with monoclonal antibodies from cell lysates. Precipitates were denatured in 2× sample buffer and analyzed by 10% SDS-PAGE followed by fluorography. Lanes represent proteins immunoprecipitated with PAb421 (Ab-1, Oncogene Science), a pan-specific anti-p53 antibody (lanes 1,4,7,10), PAb246 (Ab-4), a wild-type-specific anti-p53 antibody (lanes 2,5,8,11), or PAb240 (Ab-3), a mutant-specific anti-p53 antibody (lanes 3,6,9,12). The position of the immunoreactive p53 species is indicated by an arrow, and migrations of molecular size standards are marked.

A large percentage of human tumors with Myc deregulation exhibit mutations or deletions of p53 or overexpress Bcl-2 or Bcl-2-like proteins. In a survey of hematopoietic malignancies, tumors were found to have either p53 mutations or Bcl-2 overexpression but not both (Gaidono et al. 1991). This may reflect the common path in which these genes act so that there is no selective advantage to interfere with both mechanisms. Moreover, p53 mutations are frequently associated with tumor progression (Fearon and Vogelstein 1990; Sidransky et al. 1992; Kemp et al. 1993), perhaps reflecting a diminishment of tumor cell death due to apoptosis.

Our results show that p53 is specifically required for Myc-induced apoptosis. This is consistent with previous reports in which functional p53 is required for apoptosis induced by a variety of means in several systems. Wild-type p53 has been shown to be required for irradiation-induced apoptosis of thymocytes and for irradiation- and chemotherapeutic agent-induced apoptosis in fibroblasts sensitized by adenovirus E1A expression (Lowe et al. 1993a,b). Furthermore, reintroduction of p53 into tumor cell lines with mutant or null p53 can induce apoptosis. For example, after reintroduction of p53 into a v-Myc-induced T cell lymphoma or into a Burkitt lymphoma, apoptosis may result from overexpression of Myc in the presence of wild-type p53 (Ramqvist et al. 1993; Wang et al. 1993a). Introduction of wild-type p53 into a myeloid tumor cell line, a colon cancer cell line, and murine erythroid leukemia cells also resulted in apoptosis (Yonisch-Rouach et al. 1991; Shaw et al. 1992; Johnson et al. 1993), which may be a reflection of oncogene deregulation in these cells.

Despite the dependence of multiple forms of apoptosis on functional p53, p53 is not required for all forms of apoptosis, because phorbol ester or glucocorticoid treatment of thymocytes induces cell death even in a p53 null background (Clarke et al. 1993). In addition, androgen ablation in p53-null mice does not affect involution of the prostate (Berges et al. 1993), which has been shown to be a result of apoptosis. These examples suggest that there are multiple paths to this form of cell death. Interestingly, Bcl-2 overexpression can block both p53-dependent and p53-independent means of apoptosis, suggesting that it acts downstream of a putative junction of p53-dependent and p53-independent pathways (Alnemri et al. 1992; Fanidi et al. 1992; Lotem and Sachs 1993; Wagner et al. 1993b; Wang et al. 1993b; Chou et al. 1994; Reed 1994).

How does Myc induce apoptosis?

Myc can act as a sequence-specific transcriptional activator and directly induce the expression of certain genes (Benvenisty et al. 1992; Kretzner et al. 1992; Amin et al. 1993; Bello-Fernandez et al. 1993; Jansen-Dürr et al. 1993; Reisman et al. 1993; Wagner et al. 1993a). In addition, Myc can specifically suppress the expression of other genes (Freytag and Geddes 1992; Roy et al. 1993; Philipp et al. 1994). The ability of Myc to induce apoptosis requires dimerization with Max (Amati et al. 1993)

and the amino terminus of Myc (Evan et al. 1992), which contains domains necessary for transcriptional activation (Kato et al. 1990; Amin et al. 1993). Despite these observations suggesting transcriptional activation by Myc is a component of its ability to induce apoptosis, Myc-mediated apoptosis is not inhibited by CHX (Evan et al. 1992; A.J. Wagner and N. Hay, unpubl.), indicating that de novo protein synthesis is not essential. One possible explanation is that synthesis of a protein(s) induced by Myc and required for apoptosis escapes CHX inhibition. Other intriguing possibilities, such as direct suppression of survival genes by Myc, intimate association of Myc with DNA repair or replication machinery, or induction of noncoding RNAs capable of mediating apoptosis [as shown for tumor suppression (Hao et al. 1993; Rastinejad et al. 1993)], are conceivable.

Regardless of the mechanism by which Myc induces apoptosis, this pathway can be blocked by growth factors present in serum [IGF-1, in particular (Harrington et al. 1994)] and by survival genes such as *bcl-2*. The expression of at least one gene, *p53*, is required for the induction of this apoptotic pathway, as demonstrated by the studies presented in this report. In this respect, Myc-mediated apoptosis resembles apoptosis induced by the viral oncogene E1A. This protein stabilizes p53 (Lowe and Ruley 1993), which in turn may lead to apoptosis that can be blocked by coexpression of E1B (Debbas and White 1993). Although it was reported that Myc can induce transcription from the p53 promoter in transient transfection assays (Reisman et al. 1993), we could not demonstrate significant induction of endogenous p53 mRNA or protein following activation of MycER in serum-starved Rat 1a cells in conditions that lead to induction of ornithine decarboxylase (ODC) expression and activity and induction of cell death (Wagner et al. 1993a; A.J. Wagner and N. Hay, unpubl.). We therefore conclude that in the absence of growth factors the normal level of p53 within these cells is sufficient to confer susceptibility to Myc-mediated apoptosis. This is consistent with the observation that CHX does not block the induction of apoptosis by Myc. High levels of constitutively expressed wild-type p53, however, confer susceptibility to Myc-mediated apoptosis even in the presence of growth factors, as shown with (10.1)Val5 MycER cells.

How does p53 confer susceptibility to Myc-mediated apoptosis?

Wild-type p53 functions as a negative regulator of cell cycle progression. As discussed above, wild-type p53 can also deliver an apoptosis signal induced by a variety of cell death stimuli. At the molecular level, wild-type p53 can both activate transcription of genes through its sequence-specific DNA-binding site (Kastan et al. 1992; El-Deiry et al. 1993; Juven et al. 1993) or suppress transcription of genes that do not contain its binding site, probably through its interaction with the TATA-binding protein (Ginsberg et al. 1991; Mack et al. 1993; Ragimov et al. 1993). The function of wild-type p53 as a transcriptional activator is tightly linked to its ability to arrest

cell cycle progression (Zambetti and Levine 1993; Pietropol et al. 1994). In particular, p53 is thought to elicit growth arrest by direct induction of expression of p21^{waf1/cip1} (El-Deiry et al. 1993), a cdk inhibitor (Harper et al. 1993; Xiong et al. 1993; Dulic et al. 1994). The inhibition of cdks leads to cell cycle arrest, in part by preventing hyperphosphorylation of pRb (Pines and Hunter 1991; Reed 1992).

One possibility for how this could lead to cell death, which was suggested for E1A and E2F-mediated apoptosis, is that growth arrest signals induced by p53, at least in part by its direct induction of p21^{waf1/cip1} expression, conflict with the growth stimulus provided by Myc expression and together these signals combine to initiate a cell death cascade (Wu and Levine 1994). However, our results in conjunction with those of others raise the possibility that the abilities of p53 to induce growth arrest and to mediate apoptosis may not necessarily be coupled. First, Myc-mediated apoptosis is dependent on the presence of wild-type p53 yet does not require induction of new protein synthesis, including p21^{waf1/cip1}, by p53. This is consistent with previous results demonstrating that p53-induced apoptosis is uncoupled from p53-induced growth arrest (Yonish-Rouach et al. 1993), as well as with recent observations of Karin and colleagues indicating that UV irradiation-induced apoptosis is dependent on the presence of wild-type p53 and does not require transcriptional activation by p53 (Caelles et al. 1994). Second, isoleucine starvation, which leads to growth arrest, is capable of eliciting Myc-mediated apoptosis even in the presence of serum growth factors. It was shown that isoleucine starvation arrests cells in the G₁ phase of the cell cycle at least in part by dephosphorylation of pRB, even when p53 is sequestered by SV40 large T antigen (Münger et al. 1992). Our results demonstrate that cells that are null for wild-type p53 are refractory to Myc-mediated apoptosis elicited by isoleucine starvation. Thus, even in cells that are growth arrested in a p53-independent manner, functional p53 is still required for susceptibility to Myc-mediated apoptosis. This supports the notion that the role of p53 in apoptosis is distinct from its function in growth arrest. Finally, Myc can override the growth-arresting properties of p53 (see Fig. 4 and Table 1), arguing that a pathway towards apoptosis is separate from growth arrest because even cells overexpressing wild-type p53 are not arrested in the presence of deregulated Myc.

Taken together these results strongly suggest that the function of wild-type p53 in apoptosis is not tightly linked to its function in cell cycle arrest. Whether wild-type p53 itself serves as an intermediate player in the apoptotic pathway or plays an indirect role by suppression of survival genes remains to be elucidated.

Materials and methods

Retroviruses

To construct pMV12-MycER, the *EcoRI* fragment of pMV7-MycER (Eilers et al. 1989) was cloned into a partial *EcoRI* di-

gestion of pMV12 (Cacace et al. 1993) such that the MycER fusion gene was inserted between the 5' long terminal repeat and the thymidine kinase promoter/hygromycin phosphotransferase cassette. A DNA/lipofectamine (GIBCO/BRL) mixture was transfected into Ψ2 cells and retroviruses were prepared as described previously (Wagner et al. 1993b). Virus from the conditioned media of the Ψ2 pMV12-MycER cells was used to infect PA317 cells to produce amphotropic viruses, as described previously (Wagner et al. 1993b).

Cell culture

All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS; with certified low estrogen content, GIBCO/BRL) in a humidified atmosphere with 5% CO₂. Cells expressing ER-fusion proteins were maintained in media without phenol red (GIBCO/BRL).

MEFs were a gift of L. Donehower (Baylor College of Medicine, Houston, TX) and were prepared from 14.5-day C57/B6 embryos with homozygous inactivation of both p53 genes or from normal littermates, as described (Harvey et al. 1993). Wild-type or p53^{-/-} MEFs (at passage 5 or earlier) were infected with pMV12, pMV12-Myc, or pMV12-MycER retroviruses and selected in 400 μg/ml of hygromycin B (Calbiochem) for 48 hr and then maintained in 200 μg/ml hygromycin B. We were repeatedly unable to establish hygromycin-resistant wild-type MEFs constitutively expressing c-Myc. Wild-type MEFs expressing the MycER fusion protein were successfully established in 50 μg/ml of hygromycin B but could not be maintained as a cell line. Similar difficulties have been reported in establishing E1A-expressing MEF cell lines (Lowe et al. 1993a).

(10.1) and (10.1)Val5 cells (kindly provided by A.J. Levine, Princeton University, NJ) are immortalized mouse fibroblast cell lines from BALB/c mice (Harvey and Levine 1991; Wu et al. 1993). The (10.1) clone spontaneously deleted large portions of both p53 alleles during immortalization and does not express detectable p53; (10.1)Val5 is a derivative of (10.1) expressing a temperature-sensitive p53 protein with a valine for alanine substitution at codon 135 (Michalovitz et al. 1990). These cells were infected with pMV12 or pMV12-MycER retroviruses and selected as described above. Resistant colonies were pooled after 10–14 days of selection.

Induction and analysis of cell death

Equal numbers of wild-type or p53^{-/-} MEFs infected with control or Myc-expressing retroviruses were plated in DMEM with 10% FBS. Twenty-four hours after plating, cells were washed once with phosphate-buffered saline (PBS) and starved of serum in DMEM with 0.1% FBS. Cells were maintained in these conditions for 72 hr and then photographed. p53^{-95/-} MEFs and wild-type MEFs expressing the MycER fusion protein were starved for 24 hr in DMEM/0.1% FBS and then treated with 1 μM 17β-estradiol (E2; Sigma). Cells were photographed 72 hr after addition of E2. Cell viability was determined in triplicate by trypan blue exclusion.

(10.1) pMV12, (10.1) MycER, (10.1)Val5 pMV12, and (10.1)Val5 MycER cells were maintained in phenol red-free media at 37°C. Cells were plated at 10⁶ per 100-mm-diam. plate and allowed to attach at 37°C for 15 hr. Cells were then transferred to 33°C or 39°C, and 24 hr after temperature shift, 1 μM E2 was added to plates as indicated in the figure legends. At various times after addition of E2, cells were collected and counted for growth curves, fixed in 70% EtOH in PBS as described for flow cytometric analysis (Wagner et al. 1993b), or harvested for DNA

fragmentation analysis. Identical results were obtained when temperature shift and E2 addition were simultaneous.

For isoleucine-starvation experiments, cells were washed with PBS and refed with isoleucine-deficient RPMI 1640 media (SelectAmine, GIBCO/BRL) containing 10% dialyzed FBS and incubated at 33°C or 39°C for 60 hr. As indicated, cells were simultaneously treated with 1 μ M E2.

For CHX experiments, 10⁶ cells were plated per 100-mm-diam. plate and incubated at 39°C for 12 hr. Cells were treated with 1 μ M E2 (as necessary) starting 6 hr before temperature shift and 15 μ M CHX was added 1 hr prior to temperature shift to 33°C. Control plates were left at 39°C. Sixteen hours after temperature shift, genomic DNA was harvested from the cells and analyzed as described below.

DNA fragmentation analysis

Total genomic DNA from 5 $\times$ 10⁵ cells was prepared by overnight lysis of cells at 37°C in 10 mM Tris (pH 8.0), 100 mM NaCl, 25 mM EDTA (pH 8.0), 0.5% SDS, and 0.5 mg/ml of proteinase K. Lysates were extracted twice in 1:1 phenol/chloroform followed by chloroform; then nucleic acids were precipitated in ethanol. RNA was removed by digestion in 100 μ g of RNase A per milliliter of TE (pH 7.8) for 2 hr at 37°C. DNA was then purified by two 1:1 phenol/chloroform and one chloroform extraction, ethanol precipitated, and resuspended in 50 μ l of TE buffer (pH 7.5). Twenty microliters of total DNA was electrophoresed in a 1.5% agarose/TAE gel, stained with ethidium bromide, and photographed.

In addition to ethidium bromide staining, 100 ng of total genomic DNA was end-labeled for 1 hr at 37°C by use of terminal deoxynucleotidyl transferase (Boehringer Mannheim) and [α -³²P]ddATP (3000 Ci/mmole; Amersham) as described previously (Tilly and Hsueh 1993). The reaction was terminated by the addition of EDTA (pH 8.0), to 25 mM and yeast tRNA to 1 mg/ml. Labeled DNA was ethanol precipitated, resuspended in TE (pH 7.5) and electrophoresed in a 2% agarose/TAE gel. The gel was fixed in 10% trichloroacetic acid for 30 min, rinsed with dH₂O, and dried under vacuum overnight prior to autoradiography.

Protein analysis

Total cellular protein from 2 $\times$ 10⁵ cells was prepared by lysis in 2 $\times$ sample buffer (Laemmli 1970). Lysates were electrophoresed in 8% or 12.5% SDS-polyacrylamide gels for MycER and p21 detection, respectively. After electrophoresis, proteins were electroblotted to nitrocellulose membranes by semidry transfer. Membranes were blocked overnight at 4°C in 5% (wt/vol) non-fat milk in TBST [Tris-buffered saline (50 mM Tris, pH 7.5; 150 mM NaCl) with 0.2% (vol/vol) Tween-20]. For MycER Westerns, blots were probed with 2 μ g/ml H222 rat anti-human ER monoclonal antibody (a gift of G. Greene, University of Chicago) in TBST with 1% milk for 2 hr at room temperature, then washed 4 times, 15 min each, in TBST. Membranes were then incubated with 1 μ g/ml rabbit anti-rat Ig (Zymed) and washed as above. For p21 Westerns, blots were probed with a 1:1000 dilution of rabbit anti-human p21 polyclonal antibody (PharMingen), as described above. Antibody-antigen interactions were detected by incubation with 1:2000 horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Zymed) followed by enhanced chemiluminescence (Amersham).

For detection of p53, cells were starved of methionine for 60 min in methionine- and phenol red-free media (SelectAmine, GIBCO/BRL) containing 2% dialyzed fetal bovine serum. Cells were metabolically labeled with 200 μ Ci/ml [³⁵S]methionine

(Amersham) for 4 hr, washed three times in cold phosphate-buffered saline, scraped, pelleted, and lysed on ice in lysis buffer (150 mM NaCl; 50 mM Tris at pH 8.0; 5 mM EDTA; 1 mM DTT; 1% NP-40; 1 μ g/ml pepstatin; 1 μ g/ml leupeptin; 1 mM PMSF). Lysates were precleared by 60 min of incubation at 4°C with 5 μ g/ml of mouse IgG (Zymed) then 60 min with protein A-Sepharose (Pharmacia) preloaded with rabbit anti-mouse IgG antibody (Zymed). Proteins interacting nonspecifically were removed by pelleting the protein A-Sepharose beads. Precleared lysates were split into thirds and incubated for 2 hr at 4°C with 3 μ g/ml of PAb421 (Ab-1, Oncogene Science), a pan-specific anti-p53 antibody, PAb246 (Ab-4), a wild type-specific anti-p53 antibody, or PAb240 (Ab-3), a mutant-specific anti-p53 antibody (Harlow et al. 1981; Tan et al. 1986; Yewdell et al. 1986; Gannon et al. 1990). Lysates were then incubated with preloaded rabbit anti-mouse Ig-protein A-Sepharose complexes and incubated for 60 min at 4°C. Immunoprecipitates were washed twice in lysis buffer, once in TSA (10 mM Tris at pH 8.0; 140 mM NaCl; 0.025% sodium azide), and once in 50 mM Tris (pH 6.8), then eluted by incubating for 10 min at 95°C in 2 $\times$ sample buffer. After electrophoresis in a 10% SDS-polyacrylamide gel, the gel was soaked for 30 min in 1 M sodium salicylate, washed in dH₂O, dried, and exposed to film.

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