

Src Inhibits Adriamycin-Induced Senescence and G₂ Checkpoint Arrest by Blocking the Induction of p21waf1

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Abstract

DNA-damaging drugs stop tumor cell proliferation by inducing apoptosis, necrosis, or senescence. Cyclin-dependent kinase inhibitor p21waf1 is an important regulator of these responses, promoting senescence and preventing aberrant mitosis that leads to cell death. Because tumors expressing oncogenic tyrosine kinases are relatively resistant to DNA-damaging agents, the effects of Src on cellular responses to anticancer drug Adriamycin were investigated. Src expression increased drug survival in HT1080 fibrosarcoma cells, as measured by the colony formation assay, and strongly inhibited Adriamycin-induced senescence. Src also decreased the number of apoptotic cells while increasing the fraction of cells dying through necrosis. In addition, Src inhibited the G₂ and G₁ tetraploidy checkpoints of Adriamycin-treated cells, permitting these cells to proceed into mitosis and subsequently double their DNA content. Inhibition of senescence and G₂-G₁ checkpoints in Src-expressing cells was associated with the failure of these cells to up-regulate p21waf1 in response to Adriamycin. The failure of p21waf1 induction, despite increased expression of p53 and its binding to p21waf1 promoter, was mediated by the up-regulation of c-Myc, a negative regulator of p21waf1 transcription. Conversely, ectopic expression of p21waf1 inhibited Myc transcription in Src-expressing cells, an effect that was associated with the interaction of p21waf1 with the STAT3 transcription factor at the Myc promoter. These results reveal a complex effect of Src on cellular drug responses and provide an explanation for the effect of this oncogene on cellular drug resistance. (Cancer Res 2005; 65(19): 8927-35)

Introduction

The response of tumor cells to DNA-damaging anticancer drugs is determined by many proteins and pathways that regulate drug uptake or efflux, drug-DNA interactions, damage repair, or cellular responses to unrepaired DNA damage. The latter responses include the activation of G₁ and G₂ cell cycle checkpoints, which provide cells with the time to repair damage and prevent the entry of damaged cells into mitosis, the critical phase of the cell cycle. Damage-induced checkpoint arrest, however, may also lead to permanent cessation of cell division associated with phenotypic changes originally associated with replicative senescence. In contrast to replicative senescence, however, damage-induced

senescence (sometimes termed stress or aberrant signaling induced senescence or STASIS) is not mediated by telomere shortening (1). Cells that enter mitosis after DNA damage frequently undergo aberrant mitosis (mitotic catastrophe), which may lead either to cell death through apoptosis or necrosis or to senescence, apparently as a result of stabilization of the G₁ tetraploidy checkpoint. In the present article, we use the term senescence rather than STASIS, because this term has become established in the description of drug effects. The relationships among different cellular responses to DNA damage are illustrated in Fig. 1 (modified from ref. 1).

The role of different cellular responses in drug resistance can be studied by comparing the survival of drug-treated cells where genes regulating different responses are up-regulated or down-regulated by genetic manipulations. Such analysis has been frequently carried out through the overexpression of apoptosis-inhibiting genes. Inhibition of apoptosis was shown to diminish treatment response in some cases (such as hematopoietic malignancies), but it had little effect on drug or radiation survival as measured by clonogenic assays in many cell lines derived from human solid tumors. Under these conditions, increased induction of senescence or mitotic catastrophe compensated for the inactivation of the apoptotic program, resulting in undiminished antiproliferative effect of drug treatment (2).

Cell cycle effects of DNA damage are determined to a large extent by the tumor suppressor p53, which is activated in response to the damage, and by the cyclin/cyclin-dependent kinase (cdk) inhibitor p21waf1, which is transcriptionally induced by p53 and by p53-independent mechanisms. In the absence of p53 or p21waf1, tumor cells do not undergo G₁ arrest and show diminished G₂ checkpoint and G₁ tetraploidy checkpoint, resulting in abnormal mitosis that leads to polyploidization and cell death (3–6). Inactivation of p53 or p21waf1 also strongly diminishes the induction of senescence in drug-treated cells (4). Finally, p53 inactivation also weakens in most cases the apoptosis response, whereas p21waf1 knockout can have an opposite, proapoptotic effect. As a result of these complex effects, the effect of p53 inactivation on the survival of treatment varies greatly depending on the cell line and the damaging agent (2, 7). p21waf1 inactivation has been much less frequently analyzed, and although p21waf1 mutations are rare, its inhibitory functions can be down-regulated in tumor cells through promoter methylation or cytoplasmic relocalization (8–11). This down-regulation should affect the response of tumor cells to DNA-damaging drugs.

Tumors expressing oncogenic tyrosine kinases, such as Src or Bcr/Abl, are relatively resistant to therapeutic drugs that induce DNA damage (12). Although this has been linked to an enhanced expression of survival proteins and apoptosis escape (13), these signaling pathways may also modulate other cell cycle responses to DNA damage to induce chemoresistance. In the present study, we

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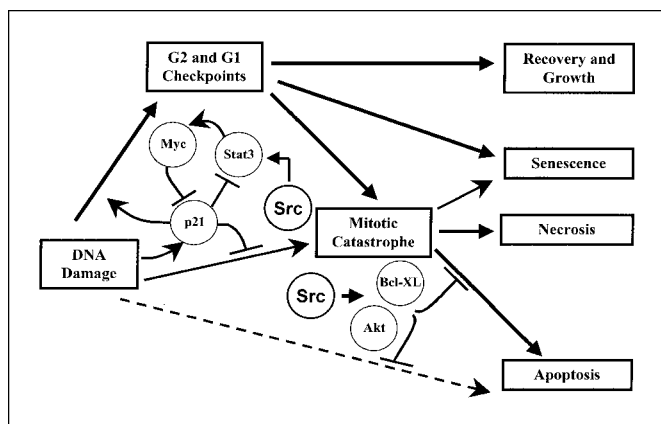


Figure 1. Cellular responses to DNA damage (modified from ref. 1) and the effects of Src-regulated proteins (see Discussion).

have investigated the effects of Src expression on the response to DNA-damaging drug Adriamycin (doxorubicin) in human HT1080 fibrosarcoma cells that are relatively resistant to damage-induced apoptosis (14, 15). As shown previously, Adriamycin-treated HT1080 cells undergo both cell death (as a consequence of mitotic catastrophe) and senescence (4). These responses to Adriamycin are regulated to a large extent by p21waf1. In particular, inhibition of p53-dependent p21waf1 induction diminishes Adriamycin-induced senescence while increasing mitotic catastrophe and apoptosis, and overexpression of p21waf1 produces the senescent phenotype (4). In the present study, we found that Src expression in HT1080 cells increased cellular resistance to Adriamycin. This effect was associated with a strong decrease in drug-induced senescence and G₂ checkpoint arrest as well as a diminution in apoptosis and an increase in necrosis. The effects of Src on cell cycle checkpoints and senescence were associated with the prevention of p21waf1 induction in drug-treated cells due to transcriptional inhibition of p21waf1 by the Myc protein, which is induced by Src. We have also found that p21waf1 inhibits Myc transcription through its interaction with the STAT3 transcription factor at the Myc promoter. These results reveal a new network of transcriptional interactions that has a profound effect on tumor cell responses to chemotherapy.

Materials and Methods

Antibodies and cell lines. Antibodies against STAT3 (C20), Src (B-12), p21waf1 (C19), Miz-1 (H190), c-Myc (N262), p53 (FL393), cyclin E (M20), cyclin D1 (H295), and phospho-histone H3 (Ser¹⁰) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-STAT3-Tyr⁷⁰⁵ were from Cell Signaling Technology (Beverly, MA). Anti- α -tubulin (T9026) was obtained from Sigma (St. Louis, MO). The fibrosarcoma cells used in this study correspond to the HT1080 p21-9 cell line that carries p21waf1 in an isopropyl-L-thio- β -D-galactopyranoside (IPTG)-inducible vector as described previously (4). Note that this cell line (referred in the text as HT1080) is p16INK4 deficient and expresses wild-type Rb and p53 (4, 16).

Cell culture, colony formation assay, synchronization, and extract preparation. For colony formation assay (Fig. 2D), 1.5×10^6 cells were plated per 10 cm plate and treated with different doses of Adriamycin for 48 hours. Cells were then washed, trypsinized, and replated in drug-free medium in six-well plates (diluted 100,000-100 cells per well) and allowed to form colonies for 14 days. For synchronization experiments, growing HT1080 cells were treated with 1 mmol/L hydroxyurea for 14 hours, washed, and further grown in RPMI with 10% fetal bovine serum for the

indicated times. The percentage of senescent cells was determined by staining SA- β -gal activity using X-gal at pH 6.0; positive cells were then counted by bright-field microscopy after scoring 100 to 1,000 cells for each sample. Nuclear extracts were prepared as described before (17). For total cell extracts, 200 μ L extraction buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μ g/mL leupeptin, 1 μ g/mL aprotinin, 1 mmol/L DTT] was added to the plates. After a 15-minute incubation on ice, total extracts were recovered by centrifugation at 12,000 rpm for 5 minutes, and extracts were either used immediately or frozen and stored at -80°C . Senescence was evaluated by measuring SA- β -gal activity as described previously (16).

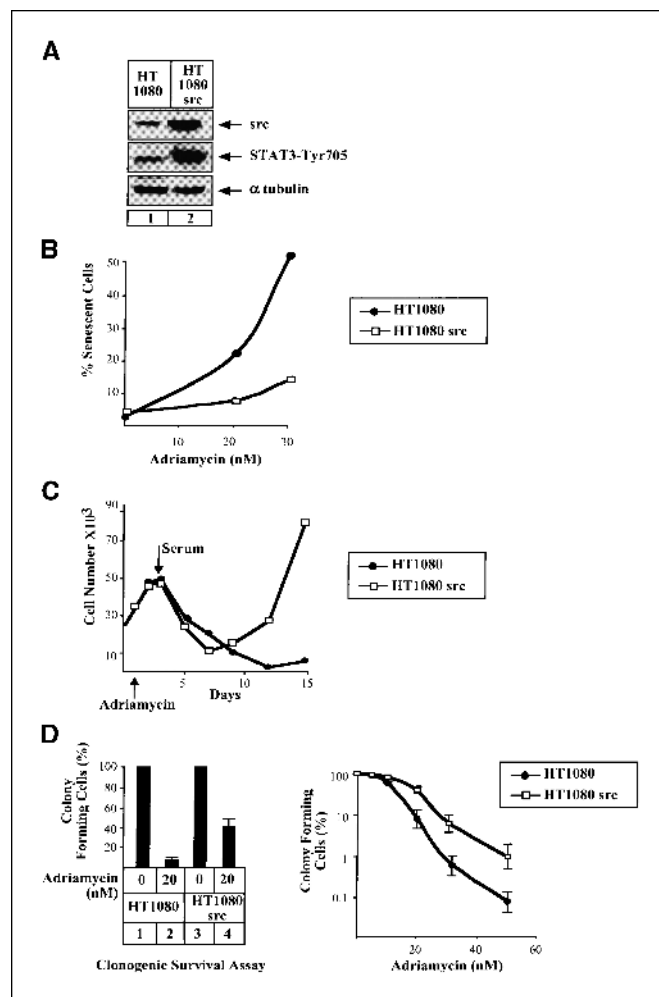


Figure 2. Src inhibits Adriamycin-induced senescence and confers resistance to Adriamycin. **A**, HT1080 cells were stably transfected with an expression vector encoding for the activated form of v-Src (HT1080-Src) or the corresponding parental plasmid (babe). Following G418 selection of 40 to 50 pooled clones, overexpression of Src was analyzed by Western blot and polyclonal antibodies against the protein (lanes 1 and 2, top). Membranes were stripped and probed with a control antibody directed against the Tyr⁷⁰⁵-phosphorylated form of STAT3 or against α -tubulin as indicated. **B**, growing cells were left untreated or treated with Adriamycin for 72 hours, fixed, and then stained with X-gal. The percentage of senescent cells was evaluated as the number of cells expressing SA- β -gal activity (representative of three experiments). **C**, cells were treated with Adriamycin (30 nmol/L) for 24 hours, washed, and then grown in 10% serum for the indicated times (representative of three experiments). **D**, HT1080 cells expressing Src or not were treated with Adriamycin for 2 days, washed, replated, and further grown for 14 days in drug-free medium as described in Materials and Methods. Colony formation was then counted using an inverted microscope, and for each cell line (expressing Src or not), growth of nontreated cells was set up at 100%. Clonogenic survival was then plotted as a fraction relative to these untreated cells.

Chromatin immunoprecipitation assay. Cells were washed and cross-linked with 1% formaldehyde at room temperature for 10 minutes. Cells were washed sequentially twice with 1 mL ice-cold PBS, centrifuged, and then resuspended in 0.5 mL lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1), 1 mmol/L PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin] and sonicated thrice for 15 seconds each at the maximum setting. Supernatants were then recovered by centrifugation at 12,000 rpm for 10 minutes at 4°C, diluted twice in dilution buffer [1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.1)], and subjected to one round of immunoclearing for 2 hours at 4°C with 2 µg sheared salmon sperm DNA, 2.5 µg preimmune serum, and 20 µL protein A-Sepharose (of 50% slurry). Immunoprecipitation was done overnight with specific antibodies; then, 2 µg sheared salmon sperm DNA and 20 µL protein A-Sepharose (of 50% slurry) were further added for 1 hour at 4°C. Immunoprecipitates were washed sequentially for 10 minutes each in TSE I [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 150 mmol/L NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 500 mmol/L NaCl], and buffer III [0.25 mol/L LiCl, 1% NP40, 1% deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8.1)]. Beads precipitates were then washed thrice with TE buffer and eluted twice with 1% SDS, 0.1 mol/L NaHCO₃. Eluates were pooled and heated at 65°C for 6 hours to reverse the formaldehyde cross-linking, and DNA was precipitated using classic procedures. For PCR, 10 µL from a 100 µL DNA preparation were used for 30 cycles of amplifications. The following regions were amplified: regions -2760/-2486, -262/-70, and -105/+25 of the p21waf1 promoter and -223/-40 (relative to P2) region of the Myc promoter.

Serial chromatin immunoprecipitation assay experiment. Following immunoprecipitation with the first STAT3 antibody, beads precipitates were prepared as described above and eluted during 4 hours at 4°C with 500 µL dilution buffer II [1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.1), 1 mmol/L PMSF, 5 mmol/L Na₃VO₄, 5 mmol/L NaF, 5-10 µg purified His-STAT3 fusion protein corresponding to amino acids 716-770]. This fusion protein is recognized by the C20 antibody and competes with the endogenous STAT3 complexes. The supernatants were diluted once to twice in dilution buffer I and immunoprecipitation was done overnight with the second specific antibody. Two micrograms of sheared salmon sperm DNA and 20 µL protein A-Sepharose (of 50% slurry) were further added for 1 hour at 4°C. Immunoprecipitates and beads were then washed as described above, eluates were pooled and heated at 65°C overnight to reverse the formaldehyde cross-linking, and DNA was then precipitated.

Flow cytometry analysis. For DNA content analysis, 2×10^5 cells were washed twice with PBS and fixed in 70% ethanol. Cells were treated with 100 units/mL RNase A for 20 minutes at 37°C, resuspended in PBS containing 50 µg/mL propidium iodide (PI), and immediately analyzed by flow cytometry. For DNA replication analysis, 2×10^5 cells were incubated with 50 µmol/L bromodeoxyuridine (BrdUrd) for 30 minutes. Cells were fixed in 70% ethanol and BrdUrd incorporation was determined by flow cytometric analysis using an anti-BrdUrd-FITC conjugate (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. For mitotic index analysis, fixed cells were incubated for 30 minutes with anti-phospho-histone H3 antibodies, washed thrice with PBS, and incubated 30 minutes with an anti-rabbit-FITC conjugate. Cells were then processed for DNA content analysis.

Confocal microscopy. Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 10 minutes and permeabilized in 70% ethanol. Cells were treated with 100 units/mL RNase A in PBS and then stained in PBS containing 50 µg/mL PI for 10 minutes. After washing, the coverslips were mounted on slides in 50% PBS/50% glycerol. Images were analyzed by confocal microscopy using a Olympus (Rungis, France) Fluoview microscope.

Results

Src inhibits Adriamycin-induced senescence and confers resistance to Adriamycin. To determine if Src confers resistance to genotoxic drugs, expression vectors encoding the oncogene (or

the vector parental form pBabe) were stably transfected into HT1080 fibrosarcoma cells that were then exposed to moderate doses of Adriamycin (10-30 nmol/L). This widely used anticancer drug produces DNA damage by stabilizing an intermediate complex formed by topoisomerase II in the process of DNA segregation. Overexpression of Src was first verified by Western blot analysis (Fig. 2A, lanes 1 and 2). Because it is well established that Src activates the STAT3 transcription factor (18, 19), we verified that STAT3 was effectively phosphorylated on its Tyr⁷⁰⁵ residue in Src-transduced cells (Fig. 2A, lanes 1 and 2). As reported previously (15), Adriamycin induced SA-β-gal activity, a specific marker of cells that do not divide or form colonies, in control cells. About 50% of the attached cells became senescent after 3 days of Adriamycin treatment (Fig. 2B). Surprisingly, the fraction of SA-β-gal-positive cells was greatly reduced in the Src-expressing population (Fig. 2B), indicating that Src inhibits drug-induced senescence. Importantly, the number of attached cells after 3 days of Adriamycin treatment was the same in Src-expressing cells and in control cells.

To analyze the effect of Src on cellular resistance to Adriamycin, we have carried out two assays. In the first assay, cells were treated with 30 nmol/L Adriamycin for 48 hours, washed, and then grown in drug-free medium for 15 days, and the cell count was determined (Fig. 1C). On transfer into drug-free medium, the number of both control and Src-expressing cells decreased to a similar degree up to day 7, indicating the death of cells on release from the drug. On subsequent days, the number of control cells decreased further without significant recovery, and almost all the cells remaining at the late time points displayed the senescent phenotype. In contrast, Src-expressing cells recovered and resumed proliferation by days 10 to 15 after release from the drug (Fig. 2C). In the second assay, we have measured colony formation after 2-day exposure to different doses of Adriamycin (Fig. 2D). In the clonogenic assay, Adriamycin showed an IC₅₀ of 12 nmol/L in control cells and 18 nmol/L in the presence of the oncogene. Moreover, we also compared the exponential shapes and initial shoulder regions of the two survival curves using the two variables, D_0 , the slope of the exponential region, and N , the extrapolational number (20). D_0 values of HT1080 and HT1080 Src were 2 and 6 nmol/L, respectively, whereas N values were 2 and 3 ($P = 0.01$). These observations suggest that Src should not be considered only as a purely dose-modifying factor but that this oncogene is probably also involved in recovery after sublethal damage. Altogether, we concluded from these results that Src confers Adriamycin resistance in HT1080 cells.

Src-expressing cells progress into mitosis and become polyploid. To characterize the effect of Src on Adriamycin-activated cell cycle checkpoints, we examined the cell cycle profile of Src-expressing cells by flow cytometry analysis. To this end, cells were arrested with hydroxyurea, released, and further treated with 30 nmol/L Adriamycin for 5 to 25 hours. Whereas control cells remained growth arrested, 15% of Src-expressing cells had already progressed into mitosis by 10 hours (Fig. 3A, left). To confirm this result, cells were treated as described above and accumulated at metaphase by nocodazole treatment. Under these conditions, 60% of Src-expressing cells accumulated into mitosis 24 hours after release, whereas control cells remained growth arrested (Fig. 3A, right). Hence, Src expression inhibits Adriamycin-induced G₂ checkpoint.

To determine if Src-expressing cells exit mitosis to undergo an additional round of DNA replication, we did BrdUrd incorporation experiments. To this end, cells were arrested as described above

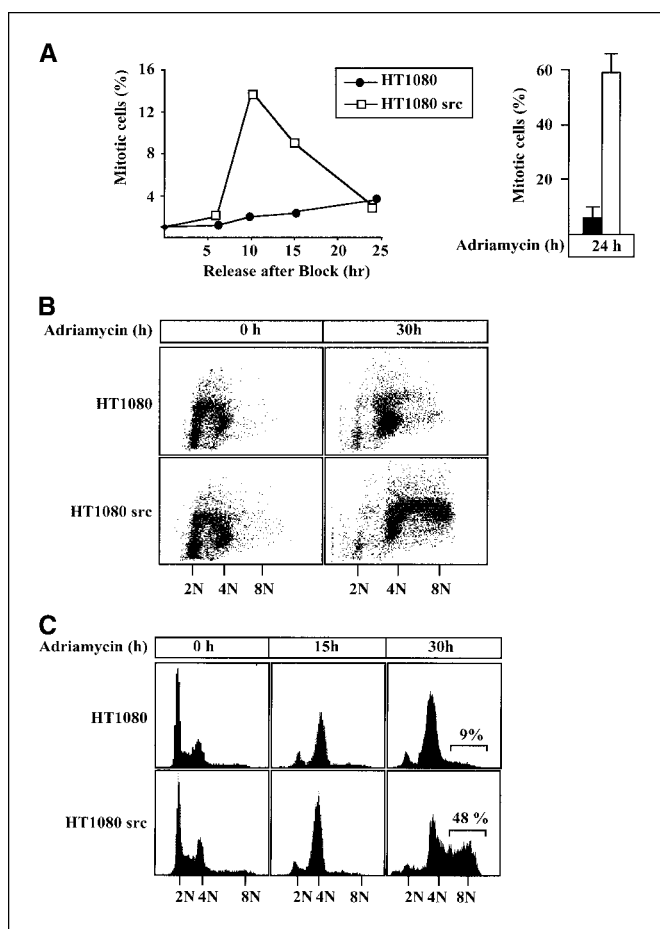


Figure 3. Src-expressing cells proceed into mitosis and become polyploid. **A**, cells were presynchronized with hydroxyurea, washed, and then either left untreated or further treated with 30 nmol/L Adriamycin for the indicated times. Fluorescence-activated cell sorting (FACS) analysis was then done on the indicated attached cells using an antibody directed against the phosphorylated form of histone H3. The same experiment was repeated in parallel, except that 20 nmol/L nocodazole was added with Adriamycin to accumulate mitotic cells (*right*). **B**, two-dimensional analysis of BrdUrd incorporation (*Y axis*, 30-minute pulse) and DNA content (*X axis*) by flow cytometry. HT1080 cells were presynchronized with hydroxyurea and treated or not with 30 nmol/L Adriamycin as indicated. **C**, cells were arrested with hydroxyurea and further treated with 30 nmol/L Adriamycin for the indicated times. FACS analysis was then done on the indicated attached cells after PI staining.

and released in the presence or absence of Adriamycin for 30 hours. As expected, most of the control cells were arrested in the fraction with 4N DNA content (Fig. 3*B*, *top*). By contrast, BrdUrd incorporation was seen in the Src population with DNA content between 4N and 8N, suggesting that these cells exit mitosis and then re-replicate their DNA (Fig. 3*B*, *bottom*). Using PI staining, we then determined if polyploid cells arose by abnormal DNA re-replication. Whereas control cells accumulated with 4N DNA content (except for ~10% of preexisting cells with >4N DNA content, note that this fraction was not modified by Adriamycin), a significant fraction (48%) of apparently polyploid nuclei was observed in Src-expressing cells (Fig. 3*C*, 3).

Altogether, we concluded from these results that the Src-expressing cells escape from G₂ arrest to proceed into mitosis, re-replicate their DNA, and become polyploid.

Src promotes mitotic catastrophe but not apoptosis. Adriamycin-induced mitotic catastrophe in HT1080 cells results

in the appearance of cells with multiple micronuclei (4, 15); such cells may proceed to apoptosis, necrosis, or senescence (Fig. 1). Interestingly, one notable feature of Src-expressing cells is an increase in the number of multinucleated cells after Adriamycin treatment. Images obtained by confocal microscopy indicated that ~80% of Src-expressing cells showed features of mitotic catastrophe at the 24-hour time point, whereas only 5% control cells were multinucleated (Fig. 4*A*, *left*). The control cells reached the same high level of multinucleation only after 80 hours. It has been recently proposed that abnormal chromosome segregation triggers apoptosis during the metaphase/anaphase transition and the G₁ tetraploidy checkpoint (21). Using PI/Annexin V staining, we observed that cell death occurred in 20% to 25% of cells 3 days after release (Fig. 4*A*, *right*). Although cell death occurred more rapidly in the presence of Src, no significant difference was observed between the two cell lines at 60 hours. To extend this result, cells were arrested with hydroxyurea and further treated with Adriamycin for 70 hours and the percentage of necrotic, apoptotic, or senescent cells was determined. Confirming the results presented in Fig. 2*B*, ~70% of control cells became senescent 3 days after release. In contrast, only 20% to 30% of Src-expressing cells stop proliferating and 40% to 50% were actively dividing in the presence of the drug (Fig. 4*B*). Interestingly, we observed that apoptosis occurred only within a minority of cells and that ~20% of Src-expressing cells were necrotic at the 70-hour time point. To further investigate the effect of Src on apoptosis, reverse transcription-PCR (RT-PCR) analysis was done to analyze the expression of proapoptotic genes. Effectively, we observed that Adriamycin treatment induced the expression of Puma and Noxa in both control and Src-expressing cells (Fig. 4*C*, *top*). However, the survival gene Bcl-XL was also constitutively activated in Src-expressing cells (Fig. 4*C*). In addition, Src also induced the constitutive activation of the Akt kinase, a well-known regulator of cell survival (Fig. 4*D*, compare *lanes 1* and *2* and *lanes 3* and *4*). By contrast, neither Bcl-XL nor Akt were found activated in control cells.

Altogether, we concluded from these results that Src-mediated G₂ checkpoint inhibition promotes mitotic catastrophe and the resultant cell death but that constitutive activation of Akt and Bcl-XL probably inhibits apoptosis in Src-expressing cells.

Src does not prevent cell cycle arrest by p21waf1 but inhibits its expression. Following Adriamycin treatment and detection of DNA damage, G₂ and G₁ tetraploidy checkpoint arrest is induced with the participation of p21waf1 (Fig. 1). As described previously (4), HT1080 cell line used in the present study (p21-9) carries p21waf1 in an IPTG-inducible vector. To determine if Src might prevent the effect of this cell cycle inhibitor on cell proliferation, we therefore induced p21waf1 by IPTG (Fig. 5*A*). As expected, Src enhanced cell proliferation and allowed growth of HT1080 cells in soft agar (Fig. 5*B*, *lanes 3* and *7*). Treatment of Src-expressing cells with IPTG to induce p21waf1 suppressed both cell proliferation and anchorage-independent growth (Fig. 5*B*, *lanes 4* and *8*). Because HT1080 cells develop morphologic markers of senescence on p21waf1 induction (4, 16), senescence induction was also analyzed in control and Src-expressing cells. To this end, cells were stained for SA- β -gal activity at pH 6. As expected, IPTG treatment efficiently induced SA- β -gal activity in control cells and this effect was observed to the same extent in the presence of Src (Fig. 5*B*, *lanes 9-12*). Hence, Src expression does not interfere with the induction of growth arrest or the senescent phenotype by p21waf1.

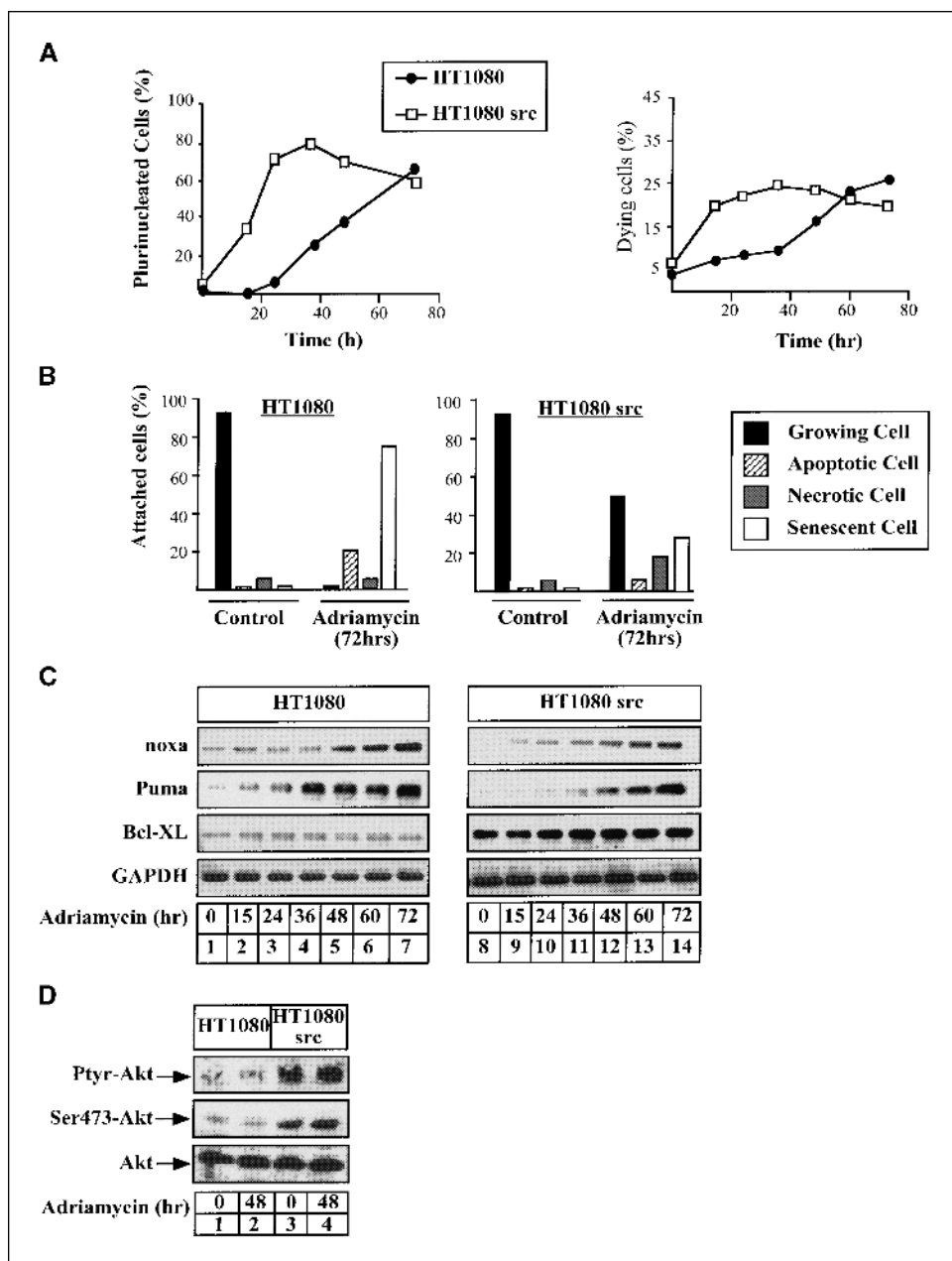
Because these findings indicated that Src does not act downstream of p21waf1, we then determined if p21waf1 was expressed to the same extent on Adriamycin treatment of control or Src-expressing cells. As expected, RT-PCR analysis indicated that p21waf1 accumulated in control cells 36 to 72 hours after drug treatment (Fig. 5C, lanes 1-8). Surprisingly, p21waf1 was not significantly expressed in Src-expressing cells (Fig. 5C, lanes 9-16). To determine if this cell cycle inhibitor binds to cyclin-cdk complexes, cells were treated with Adriamycin as described above, nuclear extracts were recovered and coimmunoprecipitations were done with polyclonal antibodies directed against p21waf1. Proteins present in the immunoprecipitates were then revealed by immunoblotting with cyclin E antibodies (Fig. 5D, lanes 1-6). In control cells and after 48 hours, we observed that p21waf1 interacts with cyclin E, suggesting that cdk2 was inhibited. As expected, no interaction was observed between cyclin E and

p21waf1 in Src-expressing cells (Fig. 5D, lanes 1-6), suggesting that cdk2 remains active despite tetraploidy of these cells. Altogether, we concluded from these results that Src prevents p21waf1 induction by DNA damage, which can explain the effects of Src on cell cycle checkpoints and senescence.

The Myc-Miz complex counteracts p21waf1 induction by p53 in Src-expressing cells. We then determined if Src prevents the expression of p21waf1 through p53, which is responsible for p21waf1 induction in drug-treated HT1080 cells (4). Using Western blot analysis, we first observed that Adriamycin induced the expression of p53 to the same extent in control and Src-expressing cells (Fig. 6A, lanes 1-4, top). Moreover, chromatin immunoprecipitation assay (ChIP) experiments indicated that p53 was recruited to the p21waf1 promoter on drug treatment in both cell lines (Fig. 6A, lanes 5-8). No amplification of a control DNA sequence unrelated to p21 was noticed (data not shown). Confirming the results of RNA analysis,

Figure 4. Adriamycin does not induce apoptosis in Src-expressing cells. A, HT1080 cells expressing Src or not were arrested by hydroxyurea, released, and further treated or not with 30 nmol/L Adriamycin for the indicated times.

The percentage of cells with multiple micronuclei was determined by confocal microscopy analysis (left), whereas cell death (apoptosis + necrosis) was evaluated in parallel by FACS analysis and Annexin V staining (right). B, cells were treated as described in (A) and the percentage of senescent cells was evaluated as the number of cells expressing SA- β -gal activity. In parallel, the number of necrotic cells (PI positive, Annexin negative) and apoptotic cells (PI negative or positive, Annexin positive) was analyzed by FACS analysis using PI/Annexin V staining. Growing cells are defined as the fraction that is neither apoptotic nor necrotic nor senescent. C, RT-PCR analysis of the expression of the Noxa, Puma, and Bcl-XL mRNAs on Adriamycin treatment of control cells (lanes 1-7) or Src-expressing cells (lanes 8-14). D, cells were arrested and treated as above and the activation of Akt was evaluated by Western blot analysis using antibodies directed against the kinase (bottom) or its Ser⁴⁷³-phosphorylated form (middle). In parallel, extracts were immunoprecipitated with a phospho-tyrosine antibody and analyzed by Western blotting to detect the tyrosine-phosphorylated form of the kinase (top).



although p53 was normally activated, p21waf1 protein was not induced in the presence of Src (Fig. 6A, lanes 1-4, middle).

We then determined if Myc is involved in the inhibition of the p21waf1 gene because this protein is well known to be activated by the Src-STAT3 pathway (19). Moreover, Myc has been shown to bind to the p21waf1 promoter to block its activation on DNA damage (22, 23). We first characterized the effect of Adriamycin on Myc expression using RT-PCR (Fig. 6B, lanes 1-4) or Western blot experiments (Fig. 6B, lanes 5-8). Although Myc expression was inhibited as expected in control cells, the gene was not significantly affected by Adriamycin in the presence of Src (Fig. 6B, lanes 3-4 and 7-8). In addition, ChIP experiments indicated that Adriamycin induced a significant association of Myc with the proximal p21waf1 promoter in Src-expressing cells (Fig. 6C, lanes 1-4). By contrast, Myc was not detected on DNA of control cells. As reported previously (24), Miz-1 (the partner of Myc) was constitutively associated with DNA and the drug slightly enhanced its DNA-binding activity (Fig. 6C, lanes 5-8). Therefore, we concluded from these results that Src inhibited p21waf1 expression by inducing the binding of Myc to its promoter.

p21waf1 prevents the expression of Myc. Because Src induces cell proliferation through STAT3 activation and Myc up-regulation (19), we then determined the effect of p21waf1 on the expression of this gene. Remarkably, Myc was strongly down-regulated when Src-expressing cells were treated with IPTG to induce ectopic p21waf1 (Fig. 7A, lanes 1-4). To confirm these results at the transcriptional level, we monitored the effect of p21waf1 on the Src-mediated induction of the Myc promoter. To this end, the HBM-Luc promoter (25) was transfected into HT1080 cells together with vectors expressing Src and p21waf1. Inclusion of a Src-expressing vector in the transfection mixture led to a 8- to 10-fold increase in reporter gene expression, but this activation was repressed by p21waf1 (Fig. 7B, lanes 1-3). Because it is well established that the STAT3 transcription factor mediates the up-regulation of Myc by Src (18, 19), we verified using ChIP experiments that STAT3 was effectively recruited to the Myc promoter and that IPTG-induced p21 did not significantly affect its DNA-binding activity (Fig. 7C, lanes 3 and 4). Interestingly, these experiments also indicated that p21waf1 was recruited to the Myc promoter in Src-expressing cells (Fig. 7C, lanes 1 and 2). Because we have shown previously that

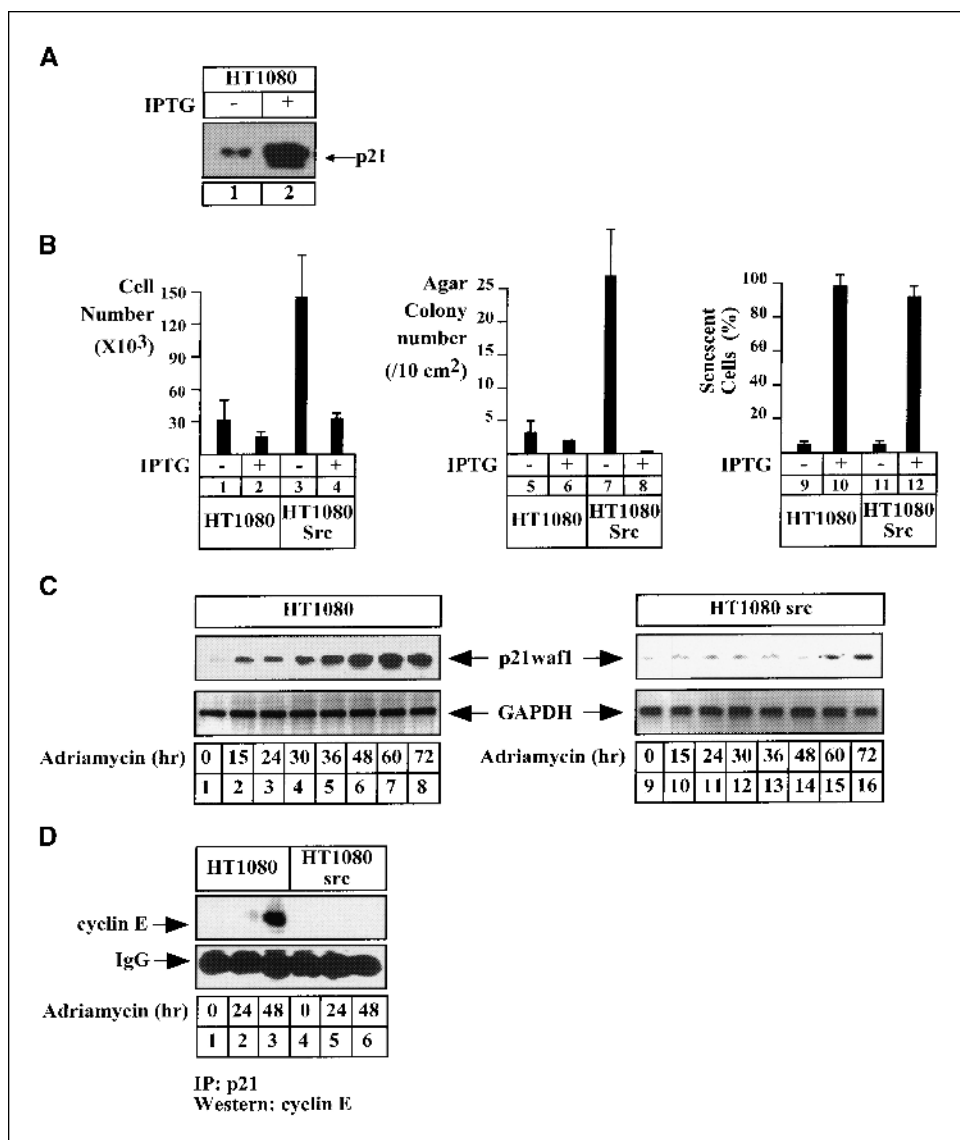


Figure 5. Src attenuates the G₁ tetraploid checkpoint through p21waf1 down-regulation. **A**, overexpression of p21waf1 on IPTG induction was verified in HT1080 cells by Western blot analysis. **B**, HT1080 cells expressing Src or not were seeded in six well-plates (20 × 10³) and their proliferation rate (lanes 1-4) and their ability to grow in soft agar (lanes 5-8) were evaluated after 4 and 10 days of culture, respectively. p21waf1 was induced on IPTG addition as indicated. In parallel, cells expressing Src or not were treated with IPTG for 24 hours, fixed, and stained with X-gal. The percentage of senescent cells was evaluated as the number of cells expressing SA-β-gal activity (lanes 9-12). **C**, RT-PCR analysis of p21waf1 expression in HT1080 cells expressing or not Src. Cells were presynchronized with hydroxyurea and further treated or not with Adriamycin for the indicated times. **D**, nuclear cell extracts were immunoprecipitated with monoclonal antibodies directed against p21waf1 proteins, separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with polyclonal antibodies directed against cyclin E.

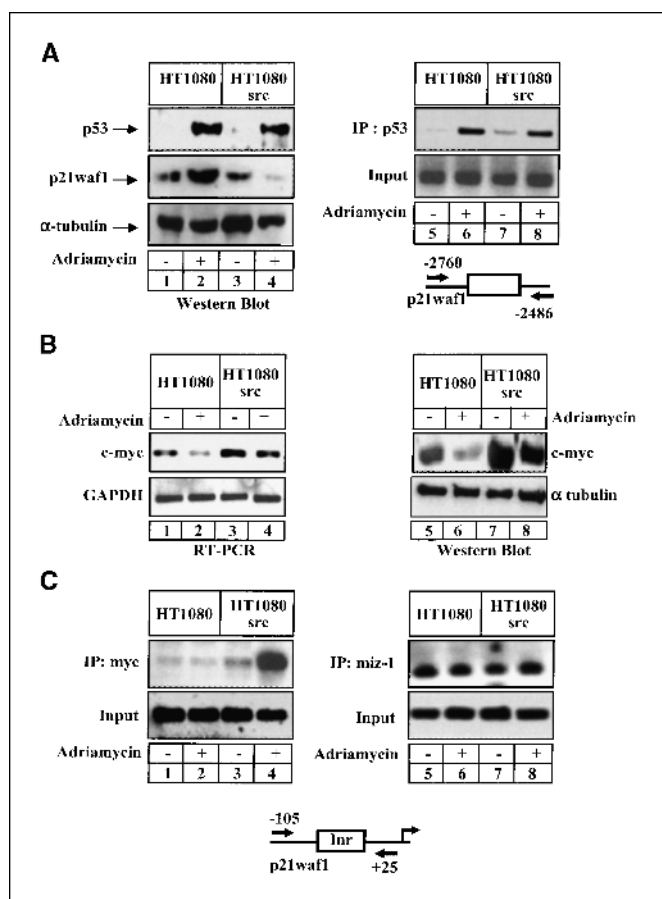


Figure 6. Adriamycin induced the association of the Myc-Miz complex with the p21waf1 promoter. *A*, asynchronously growing cells expressing (lanes 3 and 4) or not Src (lanes 1 and 2) were either left untreated or treated with Adriamycin for 48 hours (30 nmol/L). The expression of p53 was then evaluated by Western blot analysis (lanes 1-4) using polyclonal antibodies directed against the protein. Membranes were stripped and reprobed with a control antibody directed against p21waf1 or α -tubulin as indicated. In parallel, soluble chromatin was prepared from the indicated cells grown in 10% serum and treated or not with Adriamycin for 48 hours. Following immunoprecipitations with antibodies directed against p53, DNA was amplified using one pair of primers that covers the p53-binding site of the p21waf1 promoter (lanes 5-8). *B*, the indicated cells were left untreated or treated with Adriamycin for 48 hours and the expression of Myc was then analyzed by RT-PCR (lanes 1-4) or Western blot (lanes 5-8). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin expressions were monitored as controls in each experiment. *C*, soluble chromatin was prepared from the indicated cells grown in 10% serum and treated or not with Adriamycin for 48 hours. Following immunoprecipitations with antibodies directed against Myc (lanes 1-4) or Miz-1 (lanes 5-8), DNA was amplified using one pair of primers that covers the proximal p21waf1 promoter.

p21waf1 interacts with STAT3 to inhibit its activity (26), we did a serial ChIP experiment to determine if the two proteins are associated on DNA. For this, we divided the soluble chromatin derived from Src-expressing cells into two aliquots. One was immunoprecipitated with STAT3 antibodies followed by release of the immune complexes and reimmunoprecipitated with p21waf1 antibodies. The other was first immunoprecipitated with STAT3 antibodies followed by release and reimmunoprecipitated with control IgG antibodies. Under these conditions, subsequent reimmunoprecipitations with p21waf1 antibodies were able to immunoprecipitate the Myc promoter on IPTG addition, whereas this was not the case with the control IgG (Fig. 7C, lanes 5-8). In each condition, PCR analysis did not detect any occupancy of a control DNA region (data not shown).

Altogether, these results indicated that p21waf1 inhibits Myc expression, most probably by binding and inhibiting STAT3 on the Myc promoter.

Discussion

It has been proposed that certain oncogenes may provide cancer cells with an inherent mechanism of resistance to anticancer drugs (27, 28). In this study, we showed that Src oncogene has profound effects on the response of HT1080 fibrosarcoma cells to Adriamycin, and we identified the molecular mechanism of these effects. Src expression produced a substantial increase in drug resistance in these cells as measured by the colony formation assay. This overall effect was associated with a drastic decrease in the induction of senescence in drug-treated cells. On the other hand, Src abrogated drug-induced G₂ checkpoint arrest and promoted cell entry into mitosis in the presence of Adriamycin, which resulted in a more rapid onset of mitotic catastrophe, although the extent of mitotic catastrophe (as measured by the fraction of micronucleated cells) was not significantly altered in the long term. As reported by others (13), Src also diminished the apoptotic response. The antiapoptotic effect of Src, however, is counterbalanced by an increase in the fraction of cells dying through necrosis. Altogether, these results suggest that the inhibition of the senescence response is likely to be primarily responsible for increased drug resistance of Src-expressing cells.

The effects of Src on cellular drug responses and their molecular mechanisms revealed in the present study are schematized in Fig. 1. The most drastic effects of Src (i.e., the inhibition of senescence and of G₂ and G₁ tetraploidy checkpoints) can be explained by the failure of Src-expressing cells to induce p21waf1, a protein that was shown previously to be a key mediator of both G₂ (3) and G₁ tetraploidy checkpoints and drug-induced senescence (4-6). Although p21waf1 induction in response to damage in HT1080 cells is the result of transcriptional activation by p53 (4), Src expression does not affect damage-induced up-regulation of p53 or its binding to the p21waf1 promoter. In addition, several damage responses seem to be equal in control or Src-expressing cells, suggesting that Src does not diminish Adriamycin-induced DNA damage. Instead, we have found that Src prevents p21waf1 induction by activating the expression of Myc and its binding to the p21waf1 promoter, where Myc is known to act as a negative regulator of p21waf1 transcription (22, 23). Src was shown previously to induce Myc via the STAT3 transcription factor (18, 19), and here, we have confirmed that Src stimulates the binding of STAT3 to the Myc promoter.

Remarkably, we have also found that p21waf1 inhibits the expression of Myc, an effect that is apparently mediated by p21-STAT3 interactions at the Myc promoter. On cytokine stimulation, we have shown recently that STAT3 activates its target genes through its binding to histone acetyltransferases, such as NCoA1/SRC-1a or CBP. In addition, this transcription factor also associates with BRG1, the ATPase subunit of the SWI/SNF chromatin-remodeling complex, and with cdk9, the elongating kinase of the P-TEFb complex (29-31). p21waf1 is known to inhibit transcription of multiple genes through a variety of indirect mechanisms (32) and we speculate that the STAT3-p21waf1 complex confers a repressive state on chromatin to prevent the recruitment of the initiation complex and inhibit transcriptional elongation. Inhibition of Myc transcription by

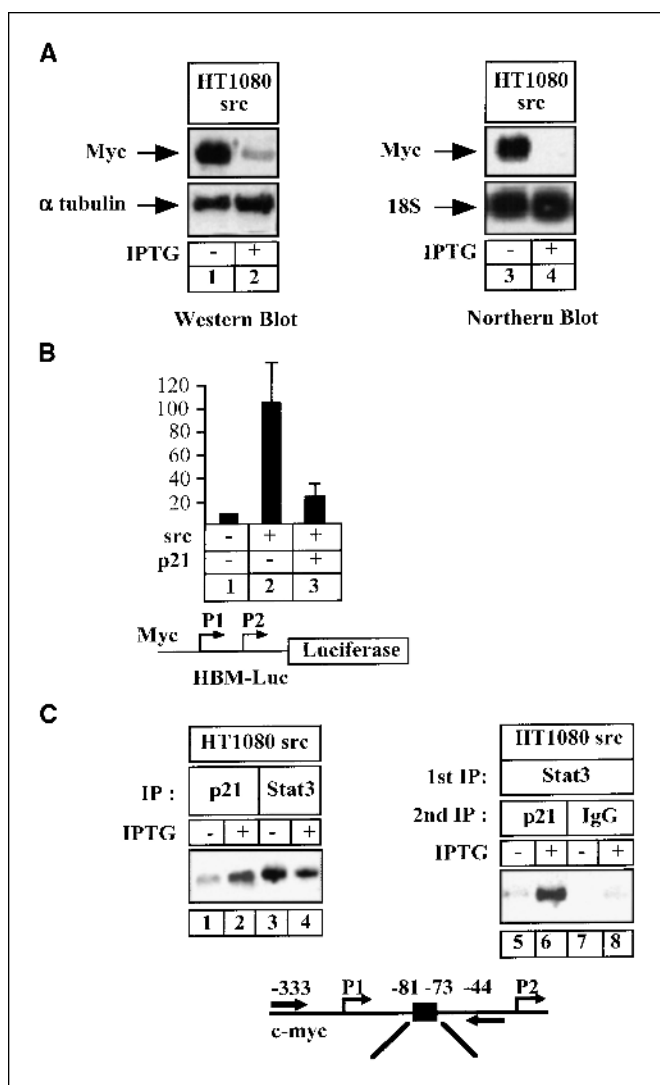


Figure 7. The expression of Myc is inhibited by p21waf1. *A*, asynchronously growing cells expressing Src were either left untreated or treated with IPTG as indicated. The expression of Myc was then analyzed by Western blot analysis (lanes 1 and 2) and tubulin expression was monitored as a control. In parallel, total RNA was prepared and 20 μ g RNA was subjected to Northern blot analysis (lanes 3 and 4) using a human Myc cDNA probe. The membrane was striped and reprobbed with a 18S oligonucleotide (bottom). *B*, HT1080 control cells were transfected with the HBM-Luc reporter gene (500 ng) in the presence or absence of vectors expressing p21waf1 or Src (500 ng). Cytoplasmic extracts were then prepared and processed to measure luciferase activity (the mean of five transfections \pm SD is shown). *C*, soluble chromatin was prepared from asynchronously growing cells treated or not with IPTG for 8 hours and immunoprecipitated with the indicated antibodies. The final DNA extractions were amplified using a pair of primer that covers the Myc proximal promoter.

p21waf1 is likely to augment p21waf1-induced cell cycle arrest, whereas inhibition of p21waf1 induction by Myc provides a positive feedback loop that amplifies the effect of the Src-Myc pathway (Fig. 1).

Figure 1 also shows that Src inhibits the apoptotic response by activating antiapoptotic proteins Bcl-XL and Akt kinase (and possibly some other proteins that were not assayed here). Although apoptosis is only a minor response to DNA damage in wild-type HT1080 cells (14, 15), the induction of antiapoptotic proteins by Src may be important to counterbalance the proapoptotic effect of Myc. Despite lower apoptosis, the overall

extent of drug-induced cell death is similar in Src-expressing and control cells, with higher levels of necrosis compensating for the decrease in apoptosis in Src-expressing cells. It is interesting to compare the effects of Src on drug response in HT1080 cells with the previously described effect of p53 inhibition, which also prevents p21waf1 induction by drug treatment (4). Although p53 inhibition also drastically decreased the induction of senescence by Adriamycin, it also produced an increase in apoptosis and overall cell death. The antiapoptotic effects of Src explain why the overall amount of cell death was not significantly enhanced, and as a result, the antisenesescence activity increased the overall clonogenic survival.

The effects of Src on cell cycle checkpoints are of special interest. Adriamycin arrests HT1080 cells preferentially in G_2 , an effect that can be explained either by the induction of G_2 checkpoint arrest as a consequence of drug-induced DNA damage or by the inhibition of topoisomerase II, the target of Adriamycin, which is required for DNA segregation during late S and G_2 . Because Src-expressing cells efficiently enter mitosis in the presence of Adriamycin, drug interaction with topoisomerase II is insufficient for G_2 arrest. The progression into mitosis of Src-expressing cells can be explained by the failure of drug-treated cells to induce p21waf1, an important contributor to the G_2 checkpoint (3). On the other hand, we have not investigated whether Src may also affect the ATM/ATR pathways, the other key regulators of the G_2 checkpoint. Remarkably however, this mitosis does not result in successful cell division and Src-expressing cells reenter the interphase without dividing and subsequently re-replicate DNA, doubling their ploidy. In normal cells, such re-replication is prevented by the G_1 tetraploidy checkpoint, which is regulated by p53 and p21waf1 (5). Besides the abrogation of the G_2 checkpoint, the effect of Src on p21waf1 expression could therefore also contribute to the failure of G_1 tetraploidy checkpoint.

An important correlation exists between STAT3 activation and oncogenic transformation by Src (18, 33, 34). Because other oncoproteins also activate the STAT3-Myc signaling pathway, our results suggest that cancer cells expressing the v-Abl, Lck, or v-Fps kinases might also be resistant to DNA-damaging drugs. Interestingly, we have already observed that the expression of p21waf1 is inhibited in glioblastoma cells (31) and the same effect has also been reported during progression of human malignant melanomas (35). Because Myc is highly expressed in glioblastoma cells due to STAT3 activation, we speculate that p21waf1 down-regulation leads to decreased senescence and increased drug resistance in these cell lines. If correct, this hypothesis would suggest that the Myc and STAT3 oncogenes cooperate to induce not only cell transformation but also drug resistance.

Acknowledgments

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References

1. Shay JW, Roninson IB. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* 2004;23:2919–33.
2. Brown JM, Wilson G. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? *Cancer Biol Ther* 2003;2:477–90.
3. Waldman T, Lengauer C, Kinzler KW, Vogelstein B. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* 1996;381:713–6.
4. Chang BD, Xuan Y, Broude EV, et al. Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene* 1999;18:4808–18.
5. Andreassen PR, Lacroix FB, Lohez OD, Margolis RL. Neither p21WAF1 nor 14-3-3 σ prevents G₂ progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21WAF1 induces stable G₁ arrest in resulting tetraploid cells. *Cancer Res* 2001;61:7660–8.
6. Andreassen PR, Lohez OD, Margolis RL. G₂ and spindle assembly checkpoint adaptation, and tetraploidy arrest: implications for intrinsic and chemically induced genomic instability. *Mutat Res* 2003;532:245–53.
7. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. *Nat Rev Cancer* 2003;3:117–29.
8. Roman-Gomez J, Castillejo JA, Jimenez A, et al. 5' CpG island hypermethylation is associated with transcriptional silencing of the p21(CIP1/WAF1/SDI1) gene and confers poor prognosis in acute lymphoblastic leukemia. *Blood* 2002;99:2291–6.
9. Allan LA, Duhig T, Read M, Fried M. The p21(WAF1/CIP1) promoter is methylated in Rat-1 cells: stable restoration of p53-dependent p21(WAF1/CIP1) expression after transfection of a genomic clone containing the p21(WAF1/CIP1) gene. *Mol Cell Biol* 2000;20:1291–8.
10. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/*neu*-overexpressing cells. *Nat Cell Biol* 2001;3:245–52.
11. Westbrook TF, Nguyen DX, Thrash BR, McCance DJ. E7 abolishes Raf-induced arrest via mislocalization of p21(Cip1). *Mol Cell Biol* 2002;22:7041–52.
12. Slupianek A, Schmutte C, Tomblin G, et al. BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance. *Mol Cell* 2001;8:795–806.
13. Johnson D, Agochiya M, Samejima K, Earnshaw W, Frame M, Wyke J. Regulation of both apoptosis and cell survival by the v-Src oncoprotein. *Cell Death Differ* 2000;7:685–96.
14. Pellegata NS, Antoniono RJ, Redpath JL, Stanbridge EJ. DNA damage and p53-mediated cell cycle arrest: a reevaluation. *Proc Natl Acad Sci U S A* 1996;93:15209–14.
15. Chang BD, Broude EV, Dokmanovic M, et al. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* 1999;59:3761–7.
16. Chang BD, Watanabe K, Broude EV, et al. Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc Natl Acad Sci U S A* 2000;97:4291–6.
17. Bienvenu F, Gascan H, Coqueret O. Cyclin D1 represses STAT3 activation through a Cdk4-independent mechanism. *J Biol Chem* 2001;276:16840–7.
18. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JEJ. Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* 1998;18:2553–8.
19. Bowman T, Broome MA, Sinibaldi D, et al. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc Natl Acad Sci U S A* 2001;98:7319–24.
20. McKenna WG, Weiss MC, Endlich B, et al. Synergistic effect of the v-myc oncogene with H-ras on radioresistance. *Cancer Res* 1990;50:97–102.
21. Castedo M, Perfettini JL, Roumier T, et al. Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* 2004;23:4362–70.
22. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 2002;419:729–34.
23. Herold S, Wanzel M, Beuger V, et al. Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* 2002;10:509–21.
24. Gartel AL, Ye X, Goufman E, et al. Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. *Proc Natl Acad Sci U S A* 2001;98:4510–5. Epub 2001 Mar 27.
25. Facchini LM, Chen S, Marhin WW, Lear JN, Penn LZ. The Myc negative autoregulation mechanism requires Myc-Max association and involves the c-myc P2 minimal promoter. *Mol Cell Biol* 1997;17:100–14.
26. Coqueret O, Gascan H. Functional interaction of STAT3 transcription factor with the cell cycle inhibitor p21WAF1/CIP1/SDI1. *J Biol Chem* 2000;275:18794–800.
27. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108:153–64.
28. Lee S, Schmitt CA. Chemotherapy response and resistance. *Curr Opin Genet Dev* 2003;13:90–6.
29. Giraud S, Bienvenu F, Avril S, Gascan H, Heery DM, Coqueret O. Functional interaction of STAT3 transcription factor with the coactivator NcoA/SRC1a. *J Biol Chem* 2002;277:8004–11.
30. Giraud S, Hurlstone A, Avril S, Coqueret O. Implication of BRG1 and cdk9 in the STAT3-mediated activation of the p21waf1 gene. *Oncogene* 2004;23:7391–8.
31. Barré B, Avril S, Coqueret O. Opposite regulation of *myc* and p21waf1 transcription by STAT3 proteins. *J Biol Chem* 2003;278:2990–6.
32. Perkins ND. Not just a CDK inhibitor: regulation of transcription by p21(WAF1/CIP1/SDI1). *Cell Cycle* 2002;1:39–41.
33. Yu CL, Meyer DJ, Campbell GS, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 1995;269:81–3.
34. Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* 1998;18:2545–52.
35. Florenes VA, Lu C, Bhattacharya N, et al. Interleukin-6 dependent induction of the cyclin dependent kinase inhibitor p21WAF1/CIP1 is lost during progression of human malignant melanoma. *Oncogene* 1999;18:1023–32.

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Src Inhibits Adriamycin-Induced Senescence and G₂ Checkpoint Arrest by Blocking the Induction of p21^{waf1}

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