Overexpression of MYC causes p53-dependent G2 arrest of normal fibroblasts

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Overexpression of the proto-oncogene MYC has been implicated in the genesis of diverse human cancers. One explanation for the role of MYC in tumorigenesis has been that this gene might drive cells inappropriately through the division cycle, leading to the relentless proliferation characteristic of the neoplastic phenotype. Herein, we report that the overexpression of MYC alone cannot sustain the division cycle of normal cells but instead leads to their arrest in G2. We used an inducible form of the MYC protein to stimulate normal human and rodent fibroblasts. The stimulated cells passed through G1 and S but arrested in G2 and frequently became aneuploid, presumably as a result of inappropriate reinitiation of DNA synthesis. Absence of the tumor suppressor gene p53 or its downstream effector p21 reduced the frequency of both G2 arrest and aneuploidy, apparently by compromising the G2 checkpoint control. Thus, relaxation of the G2 checkpoint may be an essential early event in tumorigenesis by MYC. The loss of p53 function seems to be one mechanism by which this relaxation commonly occurs. These findings dramatize how multiple genetic events can collaborate to produce neoplastic cells.

The MYC proto-oncogene encodes a transcription factor whose activity has been implicated in diverse cellular functions, including proliferation, differentiation, and apoptosis (1–3). Overexpression of MYC has been found in numerous human tumors and is thought to play a role in tumorigenesis (1). Excessive activity of MYC might contribute to tumorigenesis in at least three ways: by driving cells inappropriately through the division cycle (1, 2), by creating a mutator phenotype consequent to destabilization of the cellular genome (4–8), and by impeding cellular differentiation (1, 2).

The effect of MYC on the cell-division cycle has been examined mainly in established lines of rodent cells, which are readily transformed by oncogenes and presumably carry multiple genetic lesions. In this study, we examined the effect of MYC on the division cycle of normal rodent and human cells. To perform these studies, we used a molecular construct in which MYC is fused to the hormone-binding domain of the human estrogen receptor (MYCER; ref. 9). The chimeric gene product is active only in the presence of estradiol (E2) or hydroxytamoxifen.

Stimulation with MYC caused normal rodent and human cells to traverse the G1 and S phases of the division cycle, but the cells then arrested in G2 and frequently became aneuploid, apparently as a result of endoreduplication. Absence of the tumor suppressor protein p53 or its downstream effector p21 reduced the frequency of both G2 arrest and aneuploidy, presumably by compromising the G2 checkpoint control. Thus, relaxation of the G2 checkpoint may be an essential early event in tumorigenesis by MYC. Loss of p53 function is among the most common genetic lesions in cancer cells and represents a means by which such relaxation could occur (10, 11).

Methods

Cell Culture. Normal human fibroblasts (NHF) were derived from newborn foreskin. Mouse embryonic fibroblasts (MEF) were isolated by using conventional techniques from day 14 embryos. Cells were cultured in DMEM supplemented with 10% (vol/vol) FCS and penicillin/streptomycin. Fibroblasts were infected with preparations of the pBABE-puro retrovirus containing MYCER as described (7). Normal human fibroblasts (NHF) were derived from newborn foreskin. Mouse embryonic fibroblasts (MEF) were isolated by using conventional techniques from day 14 embryos.

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to cellular apoptosis (3). Therefore, we examined the possibility that apoptosis might account for the failure of NHF and MEF to proliferate in response to activated MYCER. We examined cells for apoptosis by phase microscopy, trypan blue exclusion, fluorescence-activated cell sorting analysis, and video microscopy. We did not detect appreciable apoptosis of either NHF or MEF in response to activation of MYCER (data not shown and Fig. 1c). By video microscopy, we observed that, on activation of MYCER, the number of mitoses did increase during the first 24 h, but cells did not divide. We conclude that MYCER arrests the cell cycle by preventing the completion of cellular division.

**Excess MYC Causes Cell-Cycle Arrest in G2.** We analyzed at what point the cells had arrested in the cell cycle by measuring their DNA content by fluorescence-activated cell sorting analysis of propidium-iodide-stained cells (data not shown) or by quantitative image analysis of Feulgen-stained cells (Fig. 2). Under normal growth conditions, greater than 80% of the NHF were

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**Fig. 1.** Excess of MYC activity inhibits the proliferation of NHF and MEF. To activate MYC activity conditionally, NHF, NHF-E6, or MEF infected with the retroviral vector alone (vector) or the retroviral vector containing MYCER were treated with E2 (1 μM or as otherwise specified). (a) Proliferation of NHF or NHF-E6 in the absence (Vector) or presence (MYCER) of excess of MYC activity. (b) Proliferation of MEF that are wild-type, p53−/−, or p21−/− in the absence (Vector) or presence (MYCER) of excess activity of MYC. For a and b, proliferation is expressed as the number of cells counted after a given time in culture, divided by the initial number of cells plated. (c) Video microscopy of NHF in the absence (Vector) or presence (MYCER) of excess of MYC activity. NHF (n = 25) with vector or MYCER were followed for 60 h after E2 treatment and examined for cumulative total number of cells, mitoses, and apoptosis. (d) Cloning efficiency of NHF in the presence of increasing levels of activity of MYC. NHF (n = 200) with MYCER were plated into 10-cm tissue culture plates and exposed to the indicated concentrations of E2. Colonies of over 50 cells were counted after 1 month of in vitro culture. (e) Cloning efficiency after 2 days of E2 NHF or NHF after 2 days of excess of MYC activity was measured by plating cells into 10-cm tissue culture plates in the absence of E2; colonies of over 50 cells were counted after 1 month. (f) p53 protein expression in NHF or NHF-E6 in the absence (–) or presence (+) of excess activity of MYC. Protein was measured by Western analysis with the monoclonal antibody pAB421 (Calbiochem). (a–e) Samples were obtained in triplicate. Mean values ± SD are shown. One of at least three experiments is shown.
in G\textsubscript{1} (Fig. 2a). In contrast, over 70% of the cells were in G\textsubscript{2} after 2 days of sustained activation of MYCER (Fig. 2b).

Because MYC is thought to stimulate entry into the cell cycle, we speculated that the activation of MYC may have greater consequences if cells were synchronized in G\textsubscript{0} through serum starvation. Indeed, we found that when cells were first synchronized in G\textsubscript{0}, the concurrent addition of serum and activation of MYCER now resulted in over 90% of the cells accumulating in G\textsubscript{2} (Fig. 2d). Thus, cells in G\textsubscript{0} may be more sensitive to the effects of MYC activity. Note that the serum treatment alone of cells previously synchronized in G\textsubscript{0} in low serum for 2 days resulted in less than 20% of cells accumulating in G\textsubscript{2} (Fig. 2c). We conclude that MYC caused cells to arrest in G\textsubscript{2}.

**Arrest in G\textsubscript{2} Is p53-Dependent.** We presumed that the arrest in G\textsubscript{2} could be attributed to activation of a checkpoint control (13). The tumor suppressor protein p53 can play a role in implementing the G\textsubscript{2} checkpoint (14–16). We found that the activation of MYCER in NHF led to an increase in the amount of p53 (Fig. 1f).

We pursued the role of p53 in the G\textsubscript{2} arrest by expressing the E6 oncogene of the human papilloma virus in NHF. The E6 protein facilitates the proteolytic destruction of p53 (17) and, accordingly, reduced the amounts of p53 in NHF, even when MYCER had been activated (Fig. 1f). The absence of p53 allowed NHF to proliferate normally even when MYCER had been activated with E2 (Fig. 1a). Similarly, activation of MYCER had no effect on the proliferation of MEF that were genetically deficient in either p53 or the cell-cycle kinase inhibitor p21, which is a downstream effector of p53 (ref. 17; Fig. 1b). Accordingly, activation of MYCER in the p53-deficient cells did not cause an abnormal accumulation of cells in G\textsubscript{2} (Fig. 2d versus f). We conclude that p53 is involved in the implementation of the G\textsubscript{2} arrest elicited by the activation of MYCER.

**Excess MYC Causes Aneuploidy.** Overexpression of MYC causes aneuploidy (4–8). Our results described above suggest a possible mechanism. MYC seems to cause cells to arrest in G\textsubscript{2}, and then may cause the reinitiation of DNA replication, resulting in endoreduplication. If this supposition were the case, we predicted that if we prevented cells from arresting in G\textsubscript{2}, then we would reduce the ability of MYC to cause aneuploidy.

Indeed, in NHF deficient in p53, the activation of MYC failed to arrest the cells in G\textsubscript{2} (as described above), and the frequency of aneuploidy was reduced by 10-fold compared with NHF with intact p53 (Fig. 2d versus f; see below Fig. 4a). However, when we examined mitotic cells, we found that MYC caused a comparable frequency of aneuploidy in NHF intact or deficient in p53 (Figs. 3 and 4a). The frequency of aneuploidy among cells in G\textsubscript{2} was also similar regardless of the status of p53 (Fig. 4a). We infer that the absence of p53, rather than preventing MYC from causing aneuploidy, reduces the pool of cells in G\textsubscript{2} that could become aneuploid. In fact, the frequency of aneuploidy caused by MYC correlated with the number of cells that were in G\textsubscript{2} (Fig. 4b).

**Discussion**

**Excess Activation of MYC Prohibits Proliferation.** The overexpression of MYC is generally thought to cause tumorigenesis by constitutively promoting cellular proliferation (1, 2). Our results demonstrate that, instead, excess MYC activation causes the proliferative arrest of NHF and MEF. The overexpression of MYC cannot of itself elicit neoplastic proliferation of otherwise
normal cells (1). Our results provide a possible explanation. When a normal cell undergoes a genetic event that causes the overexpression of MYC, the cell may be incapable of further proliferation.

Normal cells express MYC when they transit the cell-division cycle (1, 2). Indeed, MYC has been shown to induce cells to proliferate (1, 2). A possible explanation for the discordance with the results that we report herein is that physiological levels of MYC promote cellular proliferation, whereas high levels of MYC activation cause proliferative arrest. This arrest may not have been appreciated previously in experiments in which MYC was constitutively overexpressed, because cells that expressed high levels of MYC would be incapable of proliferating.

Substantial evidence suggests that MYC may be restrained from causing the malignant transformation of cells, because MYC activation induces apoptosis (3). Our results suggest that the activation of MYC in NHF and MEF causes a proliferative arrest without augmented apoptosis. Whether the activation of MYC causes proliferative arrest or induces apoptosis may depend on the particular cellular lineage, the differentiative state, and the mitogenic stimulation provided by the local microenvironment (16). In this regard, it is notable that the conditional activation of MYC in vivo was not observed to induce apoptosis in the skin (18).

Several reports document that MYC induces telomerase activity, which in turn may contribute to cellular immortalization (19–21). Our results suggest that although MYC may promote immortalization, cells are prevented from further proliferation unless cell-cycle checkpoints are first abrogated.

**MYC Causes p53-Dependent G2 Arrest.** MYC activation has been shown to cause genomic destabilization (4–8). Oncogenes may cause genomic damage by accelerating transit through the G1 and S phases of the cell cycle (13). Normal cells may withdraw from the cell-division cycle after the activation of MYC, because they suffer from DNA damage. This damage may trigger a checkpoint response. Cells may arrest in G2 rather than G1, because MYC seems to be capable of overriding the cell-cycle inhibition caused by gene products that normally would be responsible for causing cells to arrest in G1 (7). Apparently, MYC is incapable of overriding a G2 checkpoint response.

We observed that the implementation of the G2 checkpoint requires the activity of p53 as well as its downstream effector p21, because the loss of either gene product permits the proliferation of normal cells in the presence of excess MYC activity. The absence of p53 function has been shown to permit cells arrested in G2 to adapt and re-enter the cell cycle (14–16).
Tumorigenesis caused by MYC overexpression is greatly accelerated by the loss of p53 (11, 22). The loss of p53 accelerates tumorigenesis associated with MYC activation by preventing apoptosis (10, 11). Our results suggest the additional mechanism that the loss of p53 permits the continued proliferation of cells that are overexpressing MYC.

MYC Causes Aneuploidy. Previously, we described that MYC activation induces aneuploidy in NHF (7). Our results presented herein provide a possible explanation. MYC activation seems to cause the arrest of cells in G2. Cells arrested in G2 may be enforced by MYC to reinitiate DNA replication resulting in aneuploidy. This model is supported by the observation that the proportion of cells that become aneuploid on MYC activation correlates with the frequency of cells in G2 of the cell cycle (Fig. 3f). Furthermore, when the activation of MYC was prevented from causing G2 arrest through the inactivation of p53, the number of cells that became aneuploid was decreased 10-fold (Fig. 3e).

The loss of the function of p53 causes genomic destabilization (17); thus, it seemed paradoxical that its loss seemed to reduce the frequency of aneuploidy caused by MYC. However, on closer analysis, we found that the absence of p53 did not prevent MYC from causing aneuploidy but rather reduced the pool of cells accumulated in G2/M that could become aneuploid (Fig. 3e). Thus, within the subset of cells that are in the G2 or M phases of the cell cycle, the activation of MYC causes the same proportion of these populations to become aneuploid regardless of the status of p53 function (Fig. 3e). Indeed, because the loss of p53 prevents the arrest of the cell cycle, aneuploid cells would now be capable of proliferatively expanding.

Cell-Cycle Checkpoints as Surveillance Against Oncogenes. We have shown that MYC overexpression causes the proliferative arrest of normal cells. This arrest may be an important mechanism by which MYC overexpression is prevented from causing tumorigenesis. Several other oncogenes have been shown to induce the arrest of the cell-division cycle. These include RAS, RAF, MAPK, and E2F1 (23–26). In contrast to what we observed with MYC, these oncogenes induce a G1-cell-cycle arrest and cellular senescence. One reason for this difference may be that MYC is capable of overriding the checkpoints that operate during G1 but incapable of overriding the checkpoints during G2.

In addition to monitoring cells for DNA damage, cell-cycle checkpoints may generally function as surveillance mechanisms to prevent a single oncogene from initiating tumorigenesis, as has been suggested (27). Cancer is a multistep process, possibly because cells that acquire individual oncogenic events are suppressed from further malignant progression unless multiple cell-cycle checkpoints are compromised, presumably by other genetic events. Our results specifically suggest that for the activation of MYC to cause tumorigenesis, the G2 checkpoint may first have to be relaxed. The loss of p53 function may be one mechanism by which this relaxation commonly occurs (10, 11).

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