A Modest Reduction in c-Myc Expression Has Minimal Effects on Cell Growth and Apoptosis But Dramatically Reduces Susceptibility to Ras and Raf Transformation

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ABSTRACT

Deregulation of c-myc and mutation of ras genes is commonly found in many human tumors. Several lines of evidence indicate that c-Myc and oncogenic Ras cooperate in causing malignant transformation, but the mechanism of this cooperation is not understood. We set out to investigate the effect on transformation of a modest reduction in endogenous c-Myc expression, which was achieved using a c-myc heterozygous cell line constructed by targeted homologous recombination. In contrast to previous reports where c-Myc expression or activity was ablated using antisense or dominant-defective methods, use of c-myc ± cells provides a stable and homogeneous cell culture system with a precisely defined c-Myc expression level. In addition, this approach does not suffer from nonspecific artifacts such as antisense oligonucleotide toxicity or interference of dominant-defective proteins with multiple (and often undefined) target proteins. The striking and unexpected finding communicated here is that the relatively modest 50% reduction in c-Myc expression resulted in a greater than 10-fold reduction in susceptibility to transformation by oncogenic Ras or Raf proteins. This very significant defect in transformation potential cannot be explained on the basis of a generalized cell-cycle defect, because c-myc ± cells exhibit only a minimal (20%) reduction in proliferation. Genetic epistasis analysis indicated that c-Myc and Ras acted by independent pathways that converged to regulate the abundance of the cyclin-dependent kinase inhibitor protein p27Kip1. Anchorage deprivation elicited a strong up-regulation of p27, and a 50% reduction in c-Myc expression significantly compromised the ability of Ras to down-regulate p27. We propose that Ras and c-Myc signals cooperate to regulate the activity of cyclin D-Cdk4/6 complexes: the former by up-regulating the expression of cyclin D1 and the latter by affecting the activity of the complexes. Ectopic expression of cyclin A restored the transformation potential of c-myc ± cells, implicating it as a downstream genetic component in the pathway. From a therapeutic standpoint, it is of interest that, although transformation appears to be very sensitive to c-Myc expression levels, much larger reductions can be tolerated without causing any significant cell cycle defects.

INTRODUCTION

The fact that attachment to some sort of substratum is necessary for the proliferation of most normal cells and that malignant transformation reduces or eliminates this requirement was appreciated early in the history of cancer research (1–3). Anchorage deprivation of normal fibroblasts leads to the inhibition of both mRNA and protein synthesis (4), which leads to a G1-phase cell cycle arrest, but these effects become less pronounced with increasing transformation (5). Tumorigenicity in nude mice is strongly correlated with anchorage-independence (6), and a spread-out, flattened cell shape, rather than simple attachment, is required for proliferation of most nontransformed cells (7).

In most cells, loss of anchorage results in the suppression of cyclin D expression (8). In anchorage-deprived human fibroblasts and mouse NIH-3T3 cells, cyclin D1 mRNA levels were reduced severalfold, and cyclin D1 protein synthesis was almost completely inhibited, indicating that both transcriptional and translational mechanisms are involved in the down-regulation (9, 10). Constitutive expression of cyclin D1 overcame the G1 arrest observed in anchorage-deprived cells. Cyclin E expression was not significantly affected; however, cyclin E-Cdk2 complexes were not activated in response to serum stimulation (11, 12). This was attributable partly to an increase in the expression levels of the cyclin-dependent kinase inhibitor proteins p21Cip1 and p27Kip1 and partly to the absence of cyclin D1-Cdk4/6 complexes, which caused a redistribution of the p21 and p27 pools to cyclin E-Cdk2 (8). Because in anchorage-deprived cells both Cdk4/6 and Cdk2 activation is suppressed, the Rb family proteins remain unphosphorylated, and the E2F-mediated induction of several delay-response genes does not take place.

Cyclin A expression is also strongly anchorage-dependent (13, 14). The cyclin A promoter contains a motif that binds inhibitory E2F-4/p107 complexes. In attachment-deprived cells, p107 remains unphosphorylated and bound to E2F-4, thus suppressing cyclin A expression. Adhesion may also regulate cyclin A expression via an E2F-independent mechanism; anchorage-deprived NRK fibroblasts expressed cyclin D1 and exhibited Cdk2 activity, but cyclin A expression remained anchorage-dependent, and ectopic expression of cyclin A caused S-phase entry in the absence of attachment (15).

It is well established that extracellular matrix proteins such as fibronectin can trigger a rapid, transient activation of the mitogen-activated protein kinase pathway (16–18). In NIH-3T3 cells, activation of Ras in response to platelet-derived growth factor or epidermal growth factor stimulation was unaffected by anchorage deprivation, but activation of Erk was significantly reduced (19, 20). Lin et al. (19) implicated Raf-1 activation as the key step affected by matrix attachment, whereas Renshaw et al. (20) reported that Raf-1 activation was normal, but mitogen-activated protein/Erk kinase (MEK) activation was impaired. It is interesting to note that even in v-Ras- or v-Raf-transformed cells, Erk activity was decreased significantly by anchorage deprivation; however, because of the increased signaling elicited by the oncogenes, Erk activity was maintained at levels similar to that seen in nontransformed attached cells (20).

Expression of oncogenic Ras in anchorage-deprived NRK, NIH-3T3, or Rat-6 cells resulted in the induction of cyclin D1 and activation of cyclin E-Cdk2, leading to the phosphorylation of Rb family proteins, release of E2F, expression of cyclin A, and entry into S phase (21, 22). Experiments using Ras “effector loop” mutants (23) that selectively signal through only one of the major Ras targets, Raf, phosphatidylinositol-3-kinase, or Raf guanine nucleotide dissociation stimulator, showed that none of these pathways was sufficient by itself.
to elicit anchorage-independent growth. A pairwise activation of signaling pathways synergized in producing anchorage-independent growth, but the robustness of this response was highly cell line-dependent. These results suggest that individual Ras effector pathways may not uniquely mediate anchorage dependence but may rather cooperate to provide a general signal to overcome a threshold requirement, which may be different in different cell lines.

Deregulation of c-myc and mutation of ras are commonly found in many human tumors, and several lines of evidence indicate that Myc and oncogenic Ras cooperate in causing malignant transformation (24). Neither c-Myc nor oncogenic Ras alone was capable of transforming primary rodent fibroblasts, whereas together they did so effectively (25, 26). In later studies, transfection of oncogenic Ras alone elicited a conversion to a tumorigenic but not metastatic phenotype, whereas no tumors were observed in animals that received injections with c-myc-transfected cells (27). Similar phenomena have been observed with hematopoetic and epithelial cells. Although immortalized murine lymphoid cell lines can be obtained by infection of bone marrow with v-ras alone, coinfection with v-myc resulted in cell lines that grew to higher densities, had reduced dependence on feeder cells, and grew more robustly in soft agar (28). Along the same lines, transfection with c-myc and v-raf, but not with either alone, elicited efficient immortalization of p53 /- hematopoetic cells (29). In rat liver epithelial cells, transformation with both c-myc and oncogenic ras resulted in cultures that grew to much higher densities and formed 15–20 times more colonies in soft agar than cells transformed with ras alone (30).

Despite a large body of literature, very little is known about the mechanism of c-Myc and Ras interaction. Is Myc required for Ras-induced transformation? What molecular events lead to the observed synergy of Ras and c-Myc? To begin to address these questions, we developed a cell culture model system in which one or both copies of the c-myc gene were inactivated by gene targeting in an immortalized rat fibroblast cell line (31–33). In this study, we demonstrate that although c-Myc is not absolutely essential for the malignant transformation of immortalized rat fibroblasts by oncogenic Ras or Raf, a relatively modest 50% reduction in c-Myc expression results in a greater than 10-fold reduction in susceptibility to transformation. Furthermore, we show that this effect is correlated with the synergistic action of c-Myc and Ras in down-regulating high levels of p27 induced by anchorage deprivation.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Retrovirus Vectors. The TGR-1 cell line is an hprt- subclone of the Rat-1 cell line (34). The HET15 cell line is a heterozygous c-myc knockout derivative (c-myc ±) of TGR-1 (31, 32). The HO15.19 cell line is a homozygous c-myc knockout (c-myc -/-) derivative of HET15 (33). The ecotropic retrovirus packaging cell line Ψ2 (35) was obtained from Richard Mulligan (Harvard Medical School, Boston, MA). Cells were cultured in DMEM containing glutamine, pyruvate, high glucose, and 3.7 g/liter sodium bicarbonate, supplemented with 10% calf serum and penicillin/streptomycin. Cells were maintained in a 5% CO2 atmosphere at 37°C. Electroporation conditions were as described (34). All of the drug selections were performed by seeding cells at 30–40% confluence immediately before selection. v-Raf retrovirus was obtained from the American Type Culture Collection. Ha-Ras(G12V) cloned in the hygromycin-resistant retrovirus vector LXSH (36) was obtained from Dan DiMaio (Yale University, New Haven, CT); c-Myc and c-Myc (Δ106–143) retrovirus vectors were obtained from Charles Sawyers (Jonsson Comprehensive Cancer Center, University of California at Los Angeles, Los Angeles, CA) (37). Construction of LXSH vectors that express cyclin D1, cyclin E, or cyclin A has been described (38).

Soft Agar Growth Assay. Noble agar (2% w/v; DIFCO Laboratories) was autoclaved and cooled down to 45°C. The sterile agar solution was mixed with 2 × DMEM, calf serum, and water to a final concentration of 1 × DMEM, 10% calf serum, and 0.8% or 0.4% agar for the lower and upper layers, respectively, and held at 45°C. The lower layer solution (5 ml) was aliquoted into 6-cm tissue culture dishes and allowed to solidify at room temperature. Cells were trypsinized, spun through medium supplemented with 10% calf serum to neutralize the trypsin, resuspended in 0.5 ml of fresh medium, and counted in a Coulter counter. Indicated amounts of cells were rapidly mixed with 1.5 ml of the upper solution and immediately plated onto the solidified lower layer. After the upper layer had solidified, the dishes were transferred to 37°C. Plates were fed weekly by overlaying with 1.5 ml of fresh upper layer solution. Colonies were counted at the indicated times either microscopically or visually.

Methyl Cellulose Assay. The assay was performed as described by Assoian et al. (39) and modified by Kume et al. (40). Methyl cellulose (4000 centipoises; Sigma Chemical Co.) was made up to 7% (w/v) in sterile water heated to 80°C while stirring gently. When the particles were thoroughly wetted and evenly dispersed, ice cold water was added to bring the methyl cellulose concentration to 2.6% (w/v). The suspension was allowed to cool to 4°C for at least 1 h with constant gentle stirring. An equal volume of 2 × DMEM, 20% calf serum was added, and the suspension was stirred gently overnight at 4°C. This methyl cellulose/medium suspension could be stored at 4°C for several weeks. Cells were trypsinized and spun through medium supplemented with 10% calf serum to neutralize the trypsin. Cells (5 × 105 to 1 × 106) were resuspended in 10 ml of the methyl cellulose/medium suspension prewarmed to 37°C, transferred into polypropylene tubes, and incubated at 37°C for 72 h (the caps of the tubes were loosened to allow equilibration of the medium with the CO2 atmosphere). At the end of the incubation period, 40 ml of PBS were added and mixed well; cells were spun down at 1500 rpm for 5 min at 4°C and gently washed once with 10 ml of PBS. Harvested cells were resuspended in a small volume of PBS, counted, and analyzed by immunoblotting as indicated.

Immunoblotting Analysis. Cells were grown to 80–90% confluence at the indicated temperatures and were harvested by rapid lysis in Laemmli sample buffer (32). Lysates were supplemented with protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Equal loading of lanes was established as described (32), except that Coomasie Blue-stained gels were quantitated using the Gel Doc digital gel documentation system and Molecular Analyst software (Bio-Rad). Gels were transferred to Immobilon-P membranes (Millipore), and the immunoblots were probed using standard procedures (32, 41). Signals were visualized with the enhanced chemiluminescence system (Amersham) or the Super Signal R chemiluminescence system (Pierce Chemical Co.). Antibodies used were: cyclin A and cyclin D1 obtained from Upstate Biotechnology, Inc. (06-138, 06-137); cyclin E, p27, and p16 obtained from Santa Cruz Biotechnology (sc-481, sc-528, sc-1661); Ras, LA069 hybridoma cell line obtained from Quality Biotech (Camden, NJ); and Raf, PBB1 hybridoma cell line (42) obtained from Ulf Rapp (University of Würzburg, Würzburg, Germany).

RESULTS

Reduction of c-Myc Expression Interferes with Transformation of Rat-1 Fibroblasts. To investigate the role of c-Myc in malignant transformation, a c-myc heterozygous cell line (HET15), which had one copy of c-myc disrupted by gene targeting (31, 32), was infected with a retrovirus vector expressing v-Raf, and the efficiency of transformation was assessed by plating in soft agar. HET15 cells express c-Myc at half the wild-type level (31). As an additional and independent method of reducing c-Myc activity, TGR-1 cells (a subclone of the Rat-1 cell line; see “Materials and Methods”) were infected with retrovirus vectors expressing dominant-defective mutant of c-Myc (ΔA106–143), and clonal cell lines overexpressing the c-Myc proteins were obtained by G418 selection and confirmed by immunoblotting (data not shown).

Interference with intracellular c-Myc activity either by reducing the expression of the endogenous protein by 50% or by expressing dominant-defective c-Myc protein caused in both cases an approximately 10-fold reduction in the number of soft agar colonies (Fig. 1). Over-
Transformation Potential. Ectopic expression of cyclin A can elicit myc-HET15 cells were very stable, and the striking haplo-insufficient passage, and were thus not used in additional experiments. In contrast, of the dominant-defective Myc protein decreasing upon subsequent those produced by v-Raf (data not shown).

same results, although the colonies were larger and grew faster than expressing wild-type c-Myc resulted in 1.6-fold more colonies relative to the TGR-1 control. Infection with Ha-Ras(G12V) produced the same results, although the colonies were larger and grew faster than those produced by v-Raf (data not shown).

TGR-Myc(Δ106–143) cells proved to be unstable, with expression of the dominant-defective Myc protein decreasing upon subsequent passage, and were thus not used in additional experiments. In contrast, HET15 cells were very stable, and the striking haplo-insufficient c-myc transformation phenotype was highly reproducible. In repeat experiments, reductions in transformation efficiency of up to 40–50-fold were recorded (e.g., see Fig. 2).

Ectopic Expression of Cyclin A in c-myc ± Cells Restores Transformation Potential. Ectopic expression of cyclin A can elicit anchorage-independent growth in NRK and Rat-1a cell lines (15, 43). To investigate whether cyclin A can compensate for reduced c-Myc levels, HET15 and TGR-1 cells were infected with a cyclin A-expressing retrovirus vector, hygromycin-resistant clones were expanded, and cyclin A overexpression was screened by immunoblotting. Two TGR-1-derived and three HET15-derived clonal cell lines expressing moderate levels of cyclin A were established (Fig. 2C). Clonal derivatives of TGR-1 and HET15 infected with empty pLXSH retrovirus vector were established as controls. Cyclin A expression did not affect the growth rate or elicit anchorage-independent growth in any of the cell lines (data not shown).

The transformation efficiency of the various cell lines was determined in soft agar assays after infection with retrovirus vectors expressing either v-Raf or Ha-Ras(G12V). In the experiment shown in Fig. 2, the transformation potential of HET15/LXSH cells was approximately 40-fold below that of TGR/LXSH cells in response to both v-Raf and Ha-Ras(G12V). TGR/CycA cells gave somewhat fewer colonies (2.8-fold at most) than TGR/LXSH control cells. This result may be attributable to the known toxicity of cyclin A overexpression (15). In contrast, colony formation in HET15 cells was strongly rescued by the ectopic expression of cyclin A (10–30-fold relative to HET/LXSH controls). Consistent results were obtained in two independent trials of this experiment.

Cyclin A Lowers the v-Raf Expression Threshold Required for Transformation of c-myc ± Cells. To investigate whether the observed difference in the transformability of HET15 cells with or without ectopic cyclin A corresponds to a differential requirement for oncogene expression, well developed colonies were picked from soft agar, expanded into clonal cell lines, and the expression level of v-Raf protein was examined by immunoblotting. Four clones derived from HET/LXSH cells and six clones derived from each of HET/CycA-1, HET/CycA-2, and HET/CycA-3 cells were examined (Fig. 3). Although there was variability in v-Raf expression levels between individual cell lines, clones derived from HET15 cells with ectopic cyclin A clearly expressed significantly less v-Raf than the LXSH empty vector controls.

To determine whether ectopic cyclin A expression can affect the transformation potential in the complete absence of c-Myc, the c-myc −/− cell line HO15.19, in which both c-myc gene copies had been disrupted by gene targeting (33), was infected with the LXSH/Cyclin A retrovirus vector. Two cell lines, HO15A2 and HO15A4, that overexpress moderate levels of cyclin A were isolated (38). The parental HO15.19 cell line displays a slow growth phenotype (doubling time = 50 h) that was unaffected by the ectopic expression of

Fig. 1. Dependence of v-Raf transformation on c-Myc. The indicated cell lines (A–D) were infected with a v-Raf-expressing retrovirus vector and plated in soft agar (see "Materials and Methods"). After 14 days, plates were photographed, and visible colonies were counted. The experiment was repeated twice with consistent results. The mean number of colonies for each cell line expressed as percentage of TGR-1 is presented in the bar graph shown at right.

Fig. 2. Effect of ectopic cyclin A expression on transformation of c-myc ± cells with v-Raf (A) and Ha-Ras(G12V; B). The indicated cell lines were infected with retrovirus vectors and plated in soft agar, expanded into clonal cell lines, and the expression level of v-Raf protein was examined by immunoblotting. Four clones derived from HET/LXSH cells and six clones derived from each of HET/CycA-1, HET/CycA-2, and HET/CycA-3 cells were examined (Fig. 3). Although there was variability in v-Raf expression levels between individual cell lines, clones derived from HET15 cells with ectopic cyclin A clearly expressed significantly less v-Raf than the LXSH empty vector controls.
HO15A2 cells, HO15A4 cells, and HO/LXSH (empty vector) control cells were infected with either Ha-Ras(G12V) or v-Raf retrovirus vectors and tested in a soft agar assay. None of the cell lines formed colonies after 4 weeks of incubation in soft agar (data not shown). These results indicate that cyclin A, at least at the moderate levels to which it was overexpressed in these experiments, was not able to increase the transformation potential of \( c-myc \) null cells sufficiently to allow transformation by Ha-Ras(G12V) or v-Raf in this assay.

Ectopic Expression of Cyclins D1 and E in \( c-myc \) Cells Has a Minimal Effect on Restoring Transformation Potential. Activity of cyclin D1-Cdk4/6 and cyclin E-Cdk2 complexes has been shown to be required for anchorage-independent growth (10, 14). To determine whether ectopic expression of either cyclin D1 or cyclin E can increase the transformation potential of \( c-myc \) null cells, stable clonal cell lines were derived after infection of HET15 cells with LXSH retrovirus vectors expressing either cyclin D1 or E. As described above, for cyclin A cell lines, hygromycin B-resistant colonies were expanded into cell lines, and cyclin expression was determined by immunoblotting.

Five cell lines were recovered from the cyclin D1 infection, three of which expressed moderate levels of cyclin D1. The cell line that in the initial screen expressed the highest level of cyclin D1 protein grew slowly and proved unstable upon further passage (data not shown); therefore, it could not be used in the subsequent analysis. The two remaining cell lines as well as two TGR-1-derived cell lines expressing similar levels of cyclin D1 protein (Fig. 4C) were infected with either Ha-Ras(G12V) or v-Raf retrovirus vectors, and their transformation efficiency was measured in soft agar assays. One of the two HET15-derived cell lines (HET/CycD-2) exhibited an increase in transformation efficiency, whereas the other (HET/CycD-1) was not significantly different from the HET15/LXSH control cells (Fig. 4, A and B). Contamination of the HET/CycD-2 cell line with TGR-1 cells was excluded by Southern blot analysis of clones recovered from soft agar (data not shown). Both TGR-1-derived cell lines exhibited a modest but reproducible increase in soft agar colony formation. Although cyclin D1 levels in the two HET15-derived cell lines were similar, the HET/CycD-2 cell line reproducibly displayed a slightly higher level of expression. These results indicate that the window of cyclin D1 expression that can rescue transformation of \( c-myc \) cells may be very narrow; slightly lower than optimal expression does not rescue, whereas even a very modest increase above the optimum level is toxic to cell growth.

Eight HET15-derived cell lines were recovered after infection with the cyclin E retrovirus vector, but only one showed any elevation of cyclin E expression (Fig. 4D). When this cell line was infected with either Ha-Ras(G12V)-expressing or v-Raf-expressing retrovirus vectors and analyzed in soft agar assays, its transformation potential was indistinguishable from that of HET15/LXSH control cells (data not shown).

To address the influence of clonal variation on transformation potential, the cyclin D1 and E rescue experiments were repeated with large pools of infected cells. After infection of HET15 cells with the cyclin D1-expressing or cyclin E-expressing retrovirus vectors, at least 100 hygromycin B-resistant colonies were pooled and reinjected...
with either Ha-Ras(G12V)-expressing or v-Raf-expressing retrovirus vectors. The time between the harvest of the pool and reinfection was kept as short as possible to minimize the overgrowth of rapidly dividing clones. An aliquot of cells was analyzed for cyclin D1 and cyclin E expression by immunoblotting (Fig. 5B); both were found to be clearly overexpressed. However, neither cyclin D1 nor cyclin E changed significantly the transformation efficiency of HET15 cells in this experiment (Fig. 5A). In summary, these results indicate that, in contrast to cyclin A, ectopic expression of cyclins D1 and E cannot efficiently compensate for a reduction in c-Myc expression. Cyclin D1 appears to be capable of rescue in a very narrow range of expression, but the levels required for rescue are very close to those that cause inhibition of growth.

**Conclusion**

**c-Myc IS Required for Ha-Ras(G12V)-induced Down-Regulation of p27.** Anchorage deprivation has been reported to cause a robust reduction of p27 protein expression (10, 11). The *c-myc +/+* (TGR-1), *c-myc ±* (HET15), and *c-myc −/* (HO15.19) cells under investigation here all induce p27 when deprived of anchorage (Fig. 6A). Because Ha-Ras(G12V) is known to antagonize this response (44) and loss of c-Myc expression has been observed to result in elevation of p27 levels (38), it was of interest to investigate the interplay of Ha-Ras(G12V) and c-Myc in regulating p27 expression during anchorage-independent growth. TGR-1, HET15, and HO15.19 cells were infected with a Ha-Ras(G12V)-expressing retrovirus vector, selected with hygromycin B, and at least 100 colonies of each cell line were pooled together. The pools were analyzed by immunoblotting and found to express similar levels of Ha-Ras protein (Fig. 6B). The Ha-Ras(G12V)-expressing pools, along with parental TGR-1, HET15, and HO15.19 controls, were then deprived of anchorage for 72 h by culture in a methyl cellulose suspension medium (see “Materials and Methods”). After 72 h, the cells were harvested from the methyl cellulose, and p27 expression was analyzed by immunoblotting (Fig. 6C). Introduction of Ha-Ras(G12V) strongly decreased p27 expression in *c-myc +/+* cells; however, this down-regulation was almost completely absent in *c-myc ±* and −/* cells. It is noteworthy that a mere 2-fold reduction in c-Myc expression (*c-myc +/+ versus c-myc ± cells*) resulted in a significantly amplified defect in p27 down-regulation (5–10-fold). These results indicate that c-Myc and Ha-Ras(G12V) may cooperate in reducing p27 levels in anchorage-deprived cells.

To investigate whether cyclin A was acting upstream or downstream of p27 in rescuing the c-myc haplo-insufficiency, anchorage-independent HET15-derived cell lines expressing high levels of v-Raf (HLRaf-1) or low levels of v-Raf that were rescued by ectopic cyclin A (HA2Raf-5) were established from soft agar colonies (these clones were isolated in the experiment shown in Fig. 3). HLRaf-1, HA2Raf-5, and parental HET15 cells were anchorage deprived for 72 h by growth in methyl cellulose suspension medium, and cell lysates were analyzed by immunoblotting (Fig. 6D). Although p27 was up-regulated by anchorage deprivation in all of the cases, cells expressing high levels of v-Raf (HLRaf-1) displayed the lowest levels of p27. In cells expressing low levels of v-Raf in combination with ectopic cyclin A (HA2Raf-5), p27 levels were similar to those in HET15 control cells. These results suggest that the extent of p27 down-regulation during anchorage-independent growth depends on the level of v-Raf expression and that cyclin A acts downstream to allow anchorage-independent growth despite high p27 levels.

**Very High Levels of Ha-Ras(G12V) Can Allow Anchorage-Independent Growth in Complete Absence of c-Myc.** Despite the fact that Ha-Ras(G12V) failed to transform c-myc −/* (HO15.19) cells in a standard soft agar assay, the possibility remained that very high Ha-Ras(G12V) levels could effectively down-regulate p27 despite a complete absence of c-Myc. Because previous data indicated that clones expressing high enough Ha-Ras(G12V) levels would be rare, HO15.19 cells were infected with the LXS retrovirus vector expressing Ha-Ras(G12V), and a large number of hygromycin-resistant colonies were screened microscopically for signs of morphological transformation. Several candidate clones were obtained, and the expression of Ha-Ras was analyzed by immunoblotting (Fig. 7A). Three cell lines that expressed very high (HO/Ras-1), high (HO/Ras-
and/or 6-cm dish (soft agar assay). 2 soft agar assays. The indicated cell lines were grown on plastic and trypsinized. Cells were counted, and 100, 1,000, and 10,000 cells were plated/10-cm dish (focus formation assay) or 6-cm dish (soft agar assay). 2 × 10^5 TGR-1 were added to each focus assay plate to generate a monolayer on which focus formation could be scored. Foci and soft agar colonies are expressed as percentages of input (plated) cells. Actual values of plating efficiencies were as follows. Focus formation: HO/Ras-7, 0.07%; HO/Ras-8, 18.2%; and HO/Ras-1, 11.1%. Soft agar: HO/Ras-7, 0.48%; HO/Ras-8, 3.2%; and HO/Ras-1, 31.1%.

**Fig. 7.** Influence of high Ha-Ras(G12V) expression on p27 expression and anchorage-independent growth in c-myc −/− cells. A, expression of Ha-Ras protein in three clonal cell lines (HO/Ras-1, HO/Ras-7, and HO/Ras-8) derived from the c-myc −/− cell line HO15.19. B, expression of p27 protein in the HO/Ras-1, HO/Ras-7, and HO/Ras-8 cell lines in the presence or absence of anchorage. Protein expression data were obtained by immunoblotting. C, plating efficiency of HO/Ras-1, HO/Ras-7, and HO/Ras-8 cells in focus formation and soft agar assays. The indicated cell lines were grown on plastic and trypsinized. Cells were counted, and 100, 1,000, and 10,000 cells were plated/10-cm dish (focus formation assay) or 6-cm dish (soft agar assay). 2 × 10^5 TGR-1 were added to each focus assay plate to generate a monolayer on which focus formation could be scored. Foci and soft agar colonies are expressed as percentages of input (plated) cells. Actual values of plating efficiencies were as follows. Focus formation: HO/Ras-7, 0.07%; HO/Ras-8, 18.2%; and HO/Ras-1, 11.1%. Soft agar: HO/Ras-7, 0.48%; HO/Ras-8, 3.2%; and HO/Ras-1, 31.1%.

**Fig. 8.** Susceptibility to apoptosis in c-myc −/−, +/+, and −/− cells. A, the indicated cell lines were serum deprived (0.1% serum) at subconfluent conditions for the indicated times. Floating cells were recovered in the medium and counted, and the remaining adherent cells were counted by trypsinization and also counted. The number of floating cells is expressed as a percentage of total cells; the number of total cells remained constant over the duration of the assay. B, the indicated cell lines were treated with 2 μM etoposide at subconfluent conditions for 36 h in complete medium. Apoptosis was analyzed as in A. C, floating and adherent c-myc −/− and +/+ cells in A were processed for DNA laddering.

c-myc Haplo-insufficiency Affects Predominantly the Promotion of Transformation. A large body of work has identified three principal biological activities of c-Myc: promotion of proliferation, transformation, and apoptosis. Given that a 2-fold reduction in c-Myc levels leads to a very significant reduction of transformation potential, it was therefore of interest to investigate the cell cycle and apoptotic phenotypes of c-myc −/− cells. The cell cycle effects have been investigated in detail in the past (31–33); these studies showed that c-myc −/− cells display minimal cell cycle defects, with the exponential phase growth rate (doubling time) increasing at most by 20%.

Overexpression of c-Myc induces apoptosis, especially when combined with a reduction of serum supplementation (45, 46). When TGR-1 cells were incubated under subconfluent conditions in medium supplemented with 0.1% serum, 40–50% of the cells rounded up and detached within 24–36 h (Fig. 8A). Examination of the floating cells for DNA laddering indicated that they were apoptotic (Fig. 8C). Additional assays of apoptosis, such as terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling, annexin staining, or poly(ADP-ribose) polymerase cleavage, were also positive (data not shown). Likewise, apoptosis could be induced in TGR-1 cells by incubation in the presence of etoposide (Fig. 8B). c-myc −/− cells displayed a reduced apoptotic response to both serum deprivation and etoposide, but the defect was modest (less than 2-fold) under both...
conditions (Fig. 8, A and B). Interestingly, c-myc−/− cells were profoundly defective in some (but not all) of the apoptotic responses; a detailed mechanistic examination of these phenomena will be the topic of future studies. In summary, the haplo-insufficient c-Myc phenotype is restricted almost exclusively to transformation potential, namely, a greater than 10-fold defect in susceptibility to transformation by oncogenic Ras or Raf, whereas the cell cycle and apoptotic phenotypes are relatively minor (20% decrease in growth rate and less than 2-fold reduction in apoptosis).

DISCUSSION

The striking and unexpected finding communicated here is that a relatively modest 50% reduction in c-Myc expression resulted in a greater than 10-fold reduction in susceptibility to transformation by oncogenic Ras or Raf proteins. The salient findings in terms of underlying molecular mechanisms were that moderate overexpression of cyclin A restored the transformation potential of c-myc−/− cells and that reduction of c-Myc expression compromised the ability of oncogenic Ras or Raf to reverse the strong up-regulation of p27 triggered by anchorage deprivation. Stable expression of cyclin A in c-myc−/− cells increased by at least 10-fold colony formation in soft agar in response to oncogenic Ras or Raf. Furthermore, cell lines established from soft agar colonies of v-Raf-infected c-myc−/− cells expressed significantly higher levels of v-Raf than corresponding clones recovered after infection of c-myc−/− cells expressing cyclin A, indicating that expression of cyclin A lowers the requirement for a transforming oncogene.

Cyclin A expression in late G1 has been reported to be both adhesion- and c-Myc-dependent (14, 15), and ectopic expression of c-Myc restored cyclin A levels in adhesion-deprived Rat-1a fibroblasts (43). These data, as well as the results reported here, indicate that cyclin A acts downstream of c-Myc in regulating anchorage-independent growth. Whereas in NRK and Rat-1a cells expression of cyclin A alone was sufficient to elicit anchorage-independent growth (15, 43), this was not the case with the TGR-1 cells used in this report. Likewise, NIH-3T3 cells cannot be induced to grow in soft agar by cyclin A alone.7 Although both Rat-1a and TGR-1 are subclones of the original Rat-1 cell line, Rat-1a was selected specifically for its ease of transformation and is the only rodent fibroblast cell line known to be transformed by c-myc alone (47). The levels of cyclin A required to elicit anchorage-independent growth are likely to be somewhat variable in different cell lines, and deregulated cyclin A expression has been reported to be toxic by several groups (15, 48, 49). Indeed, constitutive high level expression of cyclin A has been reported only in NRK and HeLa cells (15), and all of the TGR-1-derived clones expressed only moderate levels of cyclin A.

The issue of toxicity likewise clouds the interpretation of cyclin E and D1 rescue of c-myc−/− cells. Although in two different assays overexpression of cyclins E and D1 elicited clearly different results than overexpression of cyclin A, it could be argued that TGR-1 cells are sensitive to cyclin E and D1 overexpression and that toxicity set in before rescue became possible. This interpretation is supported by the isolation of one cyclin D1 cell line in which the c-myc haploinsufficiency was clearly restored; however, the fact that despite repeated attempts only one cell line could be obtained and that in a large pool of clones cyclin D1 did not appreciably rescue indicates that such events must be rare and likely can only be elicited in a very narrow range of cyclin D1 expression. Overexpression of cyclin D1 but not of cyclin E using an inducible system was capable of supporting transient anchorage-independent growth in Rat-1 cells (50).

Inducible expression of cyclin E was reported to overcome p27-induced arrest in NIH-3T3 cells (51), but retroviral overexpression of cyclin E did not alleviate a p27-induced arrest in Rat-1 cells (52).

It is well established that anchorage deprivation triggers a strong accumulation of p27 protein (10, 11). This effect appears to be c-Myc independent, because c-myc+/+, −, and −/− cell lines all exhibited a similar increase in p27 when grown in methyl cellulose. However, whereas introduction of oncogenic Raf or Ras significantly reduced p27 levels in attachment-deprived c-myc+/+ cells, this process occurred much less efficiently in c-myc−/− cells. Several groups have recently demonstrated the importance of the Ras-Raf pathway in the down-regulation of p27 in growth factor-dependent cell cycle progression (53, 54), as well as in anchorage-deprived cells (44). The regulation of p27 was reported to be posttranscriptional, and both inhibition of translation as well as induction of degradation were implicated. Overexpression of c-Myc has also been reported to reduce p27 levels via posttranscriptional mechanisms (55–57), and c-Myc and Ras were observed to cooperate in reducing p27 levels in rat fibroblasts (58).

The strong cooperation between c-Myc and Ha-Ras(G12V) in eliciting anchorage-independent growth was closely correlated with p27 expression; a mere 2-fold reduction in c-Myc expression (c-myc+/+ versus c-myc−/− cells) resulted in a significantly amplified defect in p27 down-regulation (5–10-fold). The cooperation of c-Myc and Ras argues against these two genes acting in a linear pathway. Furthermore, the observation that high levels of Ras were capable of down-regulating p27 and causing anchorage-independent growth in the complete absence of c-Myc (c-myc−/− cells) indicates that c-Myc is not acting downstream of Ras. Although not explicitly addressed in the experiments reported here, there is no indication in the literature that c-Myc can act upstream of Ras. Taken together, the most plausible hypothesis is that oncogenic Ras and c-Myc act by independent pathways to reduce p27 levels, with the end result depending on the cumulative strength of the signal. What could be the mechanism of such cooperation?

Although the complete spectrum of Ras-signaling activities is complex and incompletely understood, one well documented effect is the transcriptional activation of cyclin D1 expression (21, 22). Ha-Ras(G12V) was also observed to significantly increase the levels of cyclin D1 in the studies reported here.8 c-Myc has been reported to regulate positively the expression of Cdk4 and Cdk-activating kinase and negatively the expression of p27 (38, 59). Thus, in c-myc−/− cells, the levels of Cdk4 and Cdk-activating kinase are down and the level of p27 is up, and although the changes in expression are modest (2–3-fold), they result in a strong (greater than 10-fold) reduction of Cdk4/6 activity.

An attractive and simple hypothesis is that Ras and c-Myc signals converge to regulate the activity of cyclin D-Cdk4/6 complexes, the former by up-regulating the expression of cyclin D1 and the latter by affecting the activity of the complexes. The cooperation of Ras and c-Myc to activate Cdk4/6 activity would then set into motion mechanisms that result in the degradation of p27 and full activation of cyclin E-Cdk2 complexes, leading eventually to the activation of cyclin A-Cdk2 complexes, which appear to be required for anchorage-independent growth. The mechanisms by which Ha-Ras(G12V) and c-Myc up-regulate cyclin D1 expression and Cdk4 activity are incompletely understood, and this model does not rule out additional downstream functions for either Ras or c-Myc. The exquisite fine tuning of these regulatory mechanisms is underscored by the existence of haploinsufficient phenotypes demonstrated in several previous studies:

7 S. Li and J. M. Sedivy, unpublished observations.
8 A. V. Bazarov and J. M. Sedivy, unpublished observations.
c-myc \pm cells had a clear delay in the phosphorylation of Rb and activation of cyclins E and A (32), and p27 \pm mice were significantly more susceptible to tumors in multiple tissues (60).

Myc and oncogenic Ras cooperate effectively to transform primary rodent fibroblasts but cannot transform normal human fibroblasts. Although immortalization of all cells requires a telomere maintenance mechanism, human cells activate telomerase very infrequently, whereas rodent cells do so quite readily (61). In human cells, overexpression of c-Myc has been reported to activate the expression of the catalytic protein subunit of telomerase (62); to what extent this is relevant for immortalization during tumor development is not known. There is no evidence that c-Myc facilitates the expression of telomerase in rodent cells or that telomerase participates in transformation (63), but it has been shown that deregulated expression of c-Myc establishes conditions favorable for the selection of immortalizing lesions in the p53 pathway (64). Rat-1 cells used in this study do not express detectable telomerase activity and presumably maintain telomeres by a telomerase-independent mechanism; this has precluded the investigation of whether loss of c-Myc function affects telomerase expression in this system.

Expression of oncogenic Ras triggers a growth arrest associated with the up-regulation of p53 and p16\(^{INK4a}\) in both rodent and human primary fibroblasts (65). Whereas interference with either the p16-Rb or the p53 pathway is sufficient to abrogate arrest in rodent cells, both pathways need to be ablated in human cells. Rat-1 cells used in this study are not inhibited by oncogenic Ras, presumably because they have lost functional expression of the cyclin-dependent kinase inhibitor p21 (66). Until recently, the lack of immortalized but nontransformed human cell lines equivalent to the NIH 3T3 rodent model (or the Rat-1 cell line used here) has seriously hampered the study of transformation-specific functions in human cells. Although the transformation of primary human cells by defined genetic steps has been recently achieved (67), the specific mechanisms responsible for the bypass of senescence, the activation of telomerase, and the requirement of transformed phenotypes such as anchorage-independent growth remain poorly delineated. However, it appears reasonable that once the growth inhibitory effects of oncogenic Ras have been bypassed by immortalization processes, Ras additionally needs to downregulate p27 expression to facilitate anchorage-independent growth. Anchorage deprivation increases the levels of p27 in both rodent and human fibroblasts (11, 12). Activated Ras or Raf can down-regulate p27 in a number of rodent cell lines (68). In one report (69), activated Raf did not down-regulate p27 in human IMR-90 fibroblasts. Whether the Myc-Ras cooperation mechanism described in this study is operational in human cells remains to be investigated. It is important to note, however, that p27 expression has been found to be reduced but not eliminated in many human tumors (70, 71) and that decreased levels of p27 correlate with poor prognosis and increased grade of many human cancers.

The observation that a 50% reduction of c-Myc expression results in a 20% decrease in proliferation but a greater than 10-fold reduction in susceptibility to oncogenic Ras transformation suggests the existence of two discrete thresholds for c-Myc activity, one involved in malignant transformation and the other in cell cycle progression. From a therapeutic standpoint, it is encouraging that, although transformation appears to be very sensitive to c-Myc expression levels, much larger reductions can be tolerated without causing significant cell cycle defects. Indeed, there have been several reports (72–74) of successful reversion of cancerous phenotypes using c-Myc antisense oligonucleotides in a variety of cell culture models, as well as in whole animal studies. These studies have mostly focused on tumor types in which c-Myc overexpression is believed to be causal to the development of malignancy. The data presented in this study suggest that targeting endogenous c-Myc expression in tumors where c-Myc is not overexpressed, but where the endogenous protein is likely cooperating with other activated oncoproteins, may be an efficacious and hitherto overlooked strategy. For example, approximately 30% of human tumors contain activated Ras, but in most ras gene expression is not deregulated, and the mutant protein is found at a normal level of expression. This study indicates that in these cases transformation elicited by activated Ras may be highly sensitive to endogenous c-Myc expression levels. Furthermore, the normal low level of c-Myc expression and/or activity may be a better therapeutic target than those situations where c-Myc expression has been deregulated.

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9 S. Wei and J. M. Sedivy, unpublished observations.


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