Myc Antagonizes Ras-mediated Growth Arrest in Leukemia Cells through the Inhibition of the Ras-ERK-p21Cip1 Pathway*

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Even though RAS usually acts as a dominant transforming oncogene, in primary fibroblasts and some established cell lines Ras inhibits proliferation. This can explain the virtual absence of RAS mutations in some types of tumors, such as chronic myeloid leukemia (CML). We report that in the CML cell line K562 Ras induces p21Cip1 expression through the Raf-MEK-ERK pathway. Because K562 cells are deficient for p15INK4a, p16INK4a, p14ARF, and p53, this would be the main mechanism whereby Ras up-regulates p21 expression in these cells. Accordingly, we also found that Ras suppresses K562 growth by signaling through the Raf-ERK pathway. Because c-Myc and Ras cooperate in cell transformation and c-Myc is up-regulated in CML, we investigated the effect of c-Myc on Ras activity in K562 cells. c-Myc antagonized the induction of p21Cip1 mediated by oncogenic H-, K-, and N-Ras and by constitutively activated Raf and ERK2. Activation of the p21Cip1 promoter by Ras was dependent on Sp1 binding sites in K562. However, mutational analysis of the p21 promoter and the use of a Gal4-Sp1 chimeric protein strongly suggest that c-Myc affects Sp1 transcriptional activity but not the binding of Sp1 to the p21 promoter. c-Myc-mediated impairment of Ras activity on p21 expression required a transactivation domain, a DNA binding region, and a Max binding region. Moreover, the effect was independent of Miz1 binding to c-Myc. Consistent with its effect on p21Cip1 expression, c-Myc rescued cell growth inhibition induced by Ras. The data suggest that in particular tumor types, such as those associated with CML, c-Myc contributes to tumorigenesis by inhibiting Ras antiproliferative activity.

The Ras family of small GTPases includes three closely related proteins, namely H-, K-, and N-Ras. Ras proteins are activated by signals originating in surface receptors and act as key components of signaling pathways by relaying signals downstream through diverse routes. Activated Ras proteins interact with signaling effectors that, in mammalian cells, include Raf kinases (c-Raf-1, A-Raf, and B-Raf), phosphatidylinositol 3-kinase (PI3K),1 RAL-guanine nucleotide exchange factors (Ral-GDS, RGL, RLF, and RGL3), NORE/RASSF1, and phospholipase Cε (1–5). Mutated versions of ras genes are found in ~30% of all human cancers. But the involvement of Ras in the upbringing of human cancer is a topic of hot debate, as the incidence of mutations and the particular isoform mutated varies greatly depending on the origin and the type of tumor. In this respect it has been an enigmatic finding that Ras mutations, particularly those in NRAS, are relatively frequent in acute myeloid leukemia (20–45% of the cases), whereas they are practically absent in chronic myeloid leukemia (CML) even in the blastic phase of the disease, despite the biological similarity between CML and acute myeloid leukemia blasts (6–8).

Whereas Ras constitutive activation induces oncogene-like transformation in immortalized fibroblasts, high Ras activity levels cause growth arrest and premature senescence in murine and human primary fibroblasts (9, 10). Ras also inhibits growth or induces differentiation in a wide array of tumor-derived cell lines (reviewed in Ref. 11). Whereas the antiproliferative response to activated Ras has been mainly studied in primary cells, it has been much less studied in transformed cells, and its relevance to human cancer remains largely unknown.

The antiproliferative effect of Ras is achieved through at least three mechanisms that impinge on cell cycle regulation (11–13). (a) H-Ras and Raf up-regulate the cyclin-dependent kinase inhibitors p16INK4a and p15INK4b, thus blocking RB phosphorylation and G1 progression. (b) Ras up-regulates p14ARF, which inactivates Mdm2, leading to p53 up-regulation. As p53 is a potent activator of the cyclin-dependent kinases inhibitor p21Cip1 (termed p21 hereafter), it is believed that p21 contributes importantly to the cell cycle arrest brought about by Ras. (c) We have previously demonstrated that Ras also induces growth arrest and p21 up-regulation in K562 cells, which lack p16INK4a and p15INK4b, p14ARF, and p53, thus revealing a third mechanism for the Ras antiproliferative effect (14).

In rodent fibroblasts it has been shown that high levels of constitutively active Raf and MEK can activate p21 expression per se, indicating that, at the least, the Ras-Raf-MEK pathway...
Myc Impairs Ras-mediated Growth Arrest and Activation of p21

ERR2/MEK1-LA vectors were described previously (43). pMLV-c-Myc and pMLV-Myc-In373 have also been described previously (44). c-Myc-In373 carries an insertion of four serines between the amino acids 373 and 374 of human c-Myc. The D106–143 and D244–273, c-Myc deletions were made by PCR from human cDNA and clones between the EcoRI and XhoI sites of the pME18S vector (45, 46), D348–439 (EcoRI-E37G), fragment was cloned between the EcoRI and XbaI sites of pME18S vector, and D1–98 was made by digesting the c-myc open reading frame with PvuII. The expression of c-Myc proteins directed by these vectors was confirmed by transfection in 293T cells. Other expression vectors used were pCDNA3-HA-E2P2 (provided by Nick Dayton, Massachusetts General Hospital, Boston, MA), pSG424-Gal4-Sp1 (provided by G. Gill, Harvard Medical School, Boston, MA) (47), and the pSG424-Gal4-E1k1 (provided by Melanie Cobb, Southwestern Medical Center, Dallas, Texas) (48).

Luciferase Assays—Cells (2 × 10⁶) were electroporated at 260 V and 1 millifarad (Bio-Rad Gene Pulse). Luciferase assays were performed using 2 μg of the reporter plasmid, 1 μg of the plasmid for Renilla luciferase (pRL-Null, Promega), and variable amounts of the expression vectors. 36 h after electroporation, the cells were lysed and the luciferase activity was determined using the Dual Luciferase reporter assay system (Promega). In all cases the firefly luciferase activity was referred to that of the Renilla luciferase. The luciferase reporters used for p21 transactivation were pStiluc (hereafter termed p21-Luc), a 680 bp upstream of the transcription initiation site (14), cyclin E-Luc (49), pGL2M4-Luc (50) (provided by R. Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA), -93Sp21-Luc (wild-type, mut2, mut3, mut4, mut5, and mut2 + 3 + 4) (51), pS5x5Gal4-Luc (52), and p22PR-p15uc plasmid (provided by Angel Pellicer, New York University Medical Center, New York, NY) (53, 54).

Immunofluorescence Analyses—K562/S cells grown on coverslips were transfected with 0.2 μg of a green fluorescence protein (GFP) vector (pEGFP-N2, Clontech) and 2 μg of Ras or p21 expression (pCEFL-p21) vectors using FuGENE6 transfection reagent (Roche Applied Science). 36 h after transfection, the cells were fixed for 10 min in 3.7% paraformaldehyde in phosphate-buffered saline. Then per cell were permeabilized with 0.5% Triton X-100 for 10 min, incubated with primary antibodies followed by secondary antibodies (Jackson ImmunoResearch Laboratories) and an antibody Texas Red-conjugated goat anti-rabbit IgG (1:150 in phosphate-buffered saline; Jackson ImmunoResearch Laboratories). Images were obtained with a confocal laser microscope (Bio-Rad MRC 1024).

Western Blots—Cells were lysed in Nonidet P-40 buffer, and 50 μg of protein were electrophoresed on SDS-PAGE gels and transferred onto nitrocellulose membranes by standard procedures. Ras proteins were detected with anti-H-Ras polyclonal antibody (C-20, Santa Cruz Biotechnology) and anti-Ras (C20, Santa Cruz Biotechnology) and an antibody Texas Red-conjugated goat anti-rabbit IgG (1:150 in phosphate-buffered saline; Jackson ImmunoResearch Laboratories). Images were obtained with a confocal laser microscope (Bio-Rad MRC 1024).

RESULTS

Myc Impairs Ras-mediated Growth Arrest and Activation of p21—We have previously located the Ras-responsive region of the human p21 promoter in a 210 bp region upstream of the transcription initiation site (14). This region contains several Sp1/3 binding sites as well as AP2 and E2F sites (55). We have used a luciferase reporter construct harboring this promoter zone (p21-Luc) to elucidate which effectors are involved in the regulation of p21 expression by Ras. We first tested the functionality of this reporter with increasing Ras dosages in luciferase assays in K562/S cells. The results (Fig. 1A) showed that p21 promoter activity increased in proportion with the amount of the Ras expression vector transfected. Taking into consideration that only nuclear p21 seems to be able to promote growth arrest (56), we next asked whether the p21 protein generated as a consequence of Ras activity was actually located in the nucleus. For this purpose, K562/S cells (an adherent K562 derivative) were transfected with H-Ras-G12V in addition to a GFP expression vector to identify transfected cells. The localization of p21, as detected by indirect immunofluorescence, showed that the p21 protein was...
K562 cells were electroporated using 1, 2, and 5 μg of p21-Luc reporter. Data correspond to mean values from four independent experiments. Error bars indicate S.E. B, K562/S cells were transiently co-transfected with 0.2 μg of GFP vector and 2 μg of empty vector (pCEFL), pCEFL-H-Ras-G12V, or pCEFL-p21 (as a positive control). The expression of p21 was detected by immunofluorescence as described under “Experimental Procedures.” 

**FIG. 1.** Ras induces p21 expression in the nuclei of K562 cells. A, K562 cells were electroporated using 1, 2, and 5 μg of H-Ras-G12V and 2 μg of p21-Luc reporter. Data correspond to mean values from four independent experiments. Error bars indicate S.E. B, K562/S cells were transiently co-transfected with 0.2 μg of GFP vector and 2 μg of empty vector (pCEFL), pCEFL-H-Ras-G12V, or pCEFL-p21 (as a positive control). The expression of p21 was detected by immunofluorescence as described under “Experimental Procedures.”

**Ras Induces p21 Expression through the RAF-MEK-ERK Pathway**—To elucidate the signaling pathway(s) through which Ras induces p21 in K562, we tested Ras effector domain mutants that specifically interact with a defined effector molecule. These mutants carry the oncogenic mutation G12V and an additional mutation in the effector domain. Thus, the mutant proteins Ras-G12V-T35S and Ras-G12V-D38E are known to signal only through Raf, whereas Ras-G12V-Y40C only activates PI3K and Ras-G12V-E37G can only signal through RAL-guanine nucleotide exchange factors or NORE (4, 39, 40). We first assessed by immunoblot that the five Ras vectors utilized, namely ERK2-MEK1, which locates to the cytoplasm, and ERK2-MEK1-AL, a mutant form in which four leucine residues in the MEK1 nuclear export signal had been mutated to alanines, thereby exhibiting a predominant localization to the nucleus (43). The predicted localization of the proteins encoded by both vectors has been confirmed previously in K562 cells (62).

Next, K562 cells were transfected with these effector-specific Ras mutants along with the p21-Luc reporter. It was found that only Ras-G12V-T35S and Ras-G12V-D38E were able to significantly activate the p21 reporter (Fig. 2B). By contrast, Ras-G12V-E37G and Ras-G12V-Y40C did not activate the p21 reporter above the levels observed in empty vector-transfected cells. We next sought to confirm the effects of Ras mutants on p21 protein expression using indirect immunofluorescence in transfected cells. In agreement with the results observed utilizing p21 promoter activation as readout, we found that only Ras-G12V and the mutants that signal through Raf (Ras-G12V-T35S and Ras-G12V-D38E) induced a significant expression of nuclear p21 (48–35% of the transfected cells), whereas Ras-G12V-D37G and Ras-G12V-Y40C only resulted in ~11% of p21-positive cells (Fig. 2C).

The former results suggested that Ras activates the p21 promoter predominantly through the Raf-MEK-ERK pathway. We sought to confirm this point by examining the effect of the MEK inhibitor PD98059 in p21 promoter activation induced by Ras. As shown in Fig. 2D, a gradual increase in PD98059 concentration resulted in a subsequent decrease in p21 promoter activation. In contrast, treatment with the PI3K inhibitor LY294002 did not impair Ras-mediated transactivation of p21 (not shown). Moreover, the incubation of Ras-G12V-transfected cells with PD98059 also reduced dramatically the number of p21-expressing cells (Fig. 2C). These results added further support to the idea that Ras induces p21 expression predominantly by activating the Raf-MEK-ERK pathway.

To further confirm this point, we tested whether activated constituents of the ERK pathway could also activate the p21 promoter in K562 cells. First, we transfected K562 with an expression vector for Raf-BXB, a constitutively active Raf 1 mutant that carries a deletion in the N-terminal regulatory domain (41). As expected, Raf-BXB also elicited a potent activation of the p21 promoter (Fig. 2E). We also tested p21 transcriptional activation dependence on ERK activity. For this purpose, we utilized vectors encoding for ERK2 fused in tandem with MEK1, a fusion that renders ERK2 constitutively active (43). Two versions were utilized, namely ERK2-MEK1, which locates to the cytoplasm, and ERK2-MEK1-AL, a mutant form in which four leucine residues in the MEK1 nuclear export signal had been mutated to alanines, thereby exhibiting a predominant localization to the nucleus (43). The predicted localization of the proteins encoded by both vectors has been confirmed previously in K562 cells (62). We found that ERK2-MEK1-AL potently activated the p21 reporter, which, on the other hand, was unaffected by the cytoplasmic ERK2-MEK1 (Fig. 2E). These results indicated that ERK was capable to induce p21 expression only upon translocation to the nucleus. Taken together, the above results strongly suggest that Ras activates p21 expression by acting through the Raf-MEK-ERK pathway.

**Effects of RAS Effector Mutants on K562 Cell Growth**—Using p21-antisense constructs, we have previously demonstrated that Ras-evoked induction of p21 is at least partially responsible for the Ras-induced growth inhibition of K562 cells (14). As we demonstrated above that Ras activates p21 expression through the Raf-ERK pathway, we next asked whether Ras also used this pathway to inhibit K562 growth. To test this possibility, we carried out clonogenic assays in K562/S cells. Cells were transfected with the different Ras mutants, and the arising colonies were stained and counted after 15 days of G418 selection. The results (Fig. 3) showed that the Ras mutants that signal only through Raf (i.e. Ras-G12V-T35S and Ras-G12V-D38E) inhibited the clonogenic growth of K562/S cells more potently than those selectively activating RAL-GDS or PI3K, although these mutants also exerted an anti-prolifer-
ative effect to some extent. In agreement with this finding, transfection with Raf-BXB also resulted in a dramatic reduction in clonogenic growth (not shown). Taken together, these results indicate that Ras induces p21 expression and growth arrest through the Raf-MEK-ERK pathway.

**Myc Inhibits Ras-mediated Activation of the p21 Promoter**—As noted in the Introduction, the interaction between Myc and Ras has not been studied in transformed cells where Ras is antiproliferative, such as K562. On the other hand, we have shown previously that Myc represses p21 expression in K562...
when induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (32). Thus we asked whether c-Myc could counteract the effect of Ras on p21 induction and growth arrest in this cellular model. First, we tested whether c-Myc was equally competent in abolishing the induction of p21 expression by the three Ras isoforms. It was found that c-Myc was able to antagonize the induction of p21 exerted by the HRAS, KRAS, and NRAS oncogenes. Interestingly, the c-Myc repressive effect was higher for N-Ras than for H- and K-Ras (Fig. 4). In the same fashion, c-Myc also abrogated p21 promoter activation by the H-Ras wild-type (Fig. 4). Even though wild-type H-Ras activated the p21 promoter less potently than its oncogenic counterpart, as described previously (14), c-Myc reduced p21 transactivation to a similar extent in both cases (40–60%) (Fig. 4, inset). Similar results were found for wild-type K- and N-Ras (not shown). In addition, we also found that c-Myc antagonized Ras- but not E2F2-mediated induction of the cyclin E promoter (not shown), ruling out an unspecific inhibitory effect of Myc in this system. Moreover, this effect was not exclusive to K562 cells, as it was also observed in two other CML-derived cell lines, KU812 and MEG-01 (not shown).

Myc Inhibits Ras-mediated Induction of p21 at a Point Downstream from ERK—We then investigated the point at which c-Myc could interfere with Ras signaling. We found that c-Myc was able to reverse p21 transactivation exerted by Raf-BXB and MEK-EE, a constitutively activated MEK mutant carrying S218E and S222E substitutions (Fig. 5A). Likewise, c-Myc could impair p21 up-regulation induced by MEK-ERK, thus suggesting that c-Myc must be interfering with Ras signals somewhere downstream from ERK. To explore this possibility further, we determined the effects of c-Myc on the transactivation of ELK. ELK is a bona fide substrate of ERK1/2 kinases and is activated for transcriptional activity upon ERK-mediated phosphorylation. To test the effect of c-Myc on ELK activation, K562 cells were transfected with a GAL-ELK fusion protein and a Gal4-luciferase reporter construct containing five GAL4 binding sites (5xGAL4-Luc). In this system, the transactivation of 5xGAL4-Luc indicates that ELK has been phosphorylated. As expected, Ras-G12V activated the ELK-directed transcription on Gal4-Luc reporter, but the co-expression of c-Myc did not alter GAL4-ELK-dependent Ras activity. This result indicates that c-Myc does not interfere with Ras-induced phosphorylation of ELK (Fig. 5B). We conclude from these data that c-Myc impairs the induced transactivation of the p21 gene acting downstream of ERK activity, presumably at a point that does not impede the phosphorylation of transcription factors by nucleus-translocated ERKs.

Ras-mediated Induction of p21 Promoter Depends on Sp1 Sites, and c-Myc Impairs Ras-mediated Activation of Sp1—It has been shown previously that several proximal Sp1/3 sites within the p21 promoter are required for Ras-mediated activation (51) (Fig. 6A). Also, it has been reported that the Sp1 transcription factor is essential for the repression of the p21 promoter by Myc in mouse fibroblasts and that Sp1 and c-Myc can physically interact (63). Thus, we investigated whether c-Myc required the participation of Sp1 to inhibit Ras-induced activation of the p21 promoter. To do this, we analyzed Myc-Ras interaction on a series of mutants in which each of the Sp1/3 sites at the p21 promoter have been mutated (Fig. 6A). The results showed that Sp1 sites 2 and 5 are important for Ras-induced transactivation of the p21 promoter in K562 cells (Fig. 6B), although the Sp1-3 and Sp1-4 sites also contribute to Ras-mediated activation, confirming previous results in COS-7 cells (51). Interestingly, c-Myc was able to antagonize Ras activity regardless of the functionality of all the distinct Sp1 sites (Fig. 6B). For example, the reporters with mutated Sp1-3 and Sp1-4 sites were activated by Ras only to 58 and 30%, respectively, in regard to the wild-type promoter, but c-Myc further reduced this activation by ~40% in each case. We next asked whether c-Myc was impairing Sp1 binding to the p21 promoter or Sp1 transactivation by Ras. To this purpose, we took advantage of a GAL4-Sp1 fusion protein where the GAL4 DNA binding domain substitutes for the original Sp1 DNA binding domain. The effect of c-Myc co-expression on the activation of a 5xGAL4-luciferase reporter mediated by Ras was tested in K562. The results show that Ras potently activated the transcriptional activity of Sp1 and that c-Myc inhibited the Ras-mediated activation of Sp1 (Fig. 6C). The extent of the inhibition brought about by c-Myc in this system was similar to that observed on the p21 promoter (~60%). Taken together, the results are consistent with a model where Myc impaired Ras activity at a point upstream of Sp1 binding to the promoter.

Miz1 Is Not Required for c-Myc Antagonistic Effects on Ras-mediated Induction of p21—It has been reported previously that c-Myc down-regulated p21 transcription through interaction with the zinc-finger protein Miz1, forming a complex on the p21 proximal promoter (30, 31). Miz1 sites in the p21 promoter do not overlap with Sp1/3 sites (Fig. 6A). Moreover, we have shown that c-Myc antagonizes 12-O-tetradecanoylphorbol-13-acetate-mediated up-regulation of p21 expression in a Miz1-dependent manner in K562 cells (32). Thus, we asked whether Myc-Miz1 interaction was also required for the impairment of Ras-induced p21 transactivation. We approached this question by using the c-Myc mutant c-Myc-V394D. This amino acid substitution at the Myc C-terminal ends Myc-Ras interaction on a series of mutants in which the c-Myc required the participation of Sp1 to inhibit Ras-induced activation of the p21 promoter. To do this, we analyzed Myc-Ras interaction on a series of mutants in which each of the Sp1/3 sites at the p21 promoter have been mutated (Fig. 6A). The results showed that Sp1 sites 2 and 5 are important for Ras-induced transactivation of the p21 promoter in K562 cells (Fig. 6B), although the Sp1-3 and Sp1-4 sites also contribute to Ras-mediated activation, confirming previous results in COS-7 cells (51). Interestingly, c-Myc was able to antagonize Ras activity regardless of the functionality of all the distinct Sp1 sites (Fig. 6B). For example, the reporters with mutated Sp1-3 and Sp1-4 sites were activated by Ras only to 58 and 30%, respectively, in regard to the wild-type promoter, but c-Myc further reduced this activation by ~40% in each case. We next asked whether c-Myc was impairing Sp1 binding to the p21 promoter or Sp1 transactivation by Ras. To this purpose, we took advantage of a GAL4-Sp1 fusion protein where the GAL4 DNA binding domain substitutes for the original Sp1 DNA binding domain. The effect of c-Myc co-expression on the activation of a 5xGAL4-luciferase reporter mediated by Ras was tested in K562. The results show that Ras potently activated the transcriptional activity of Sp1 and that c-Myc inhibited the Ras-mediated activation of Sp1 (Fig. 6C). The extent of the inhibition brought about by c-Myc in this system was similar to that observed on the p21 promoter (~60%). Taken together, the results are consistent with a model where Myc impaired Ras activity at a point upstream of Sp1 binding to the promoter.
moter, c-Myc-V394D was unable to reduce Ras-mediated transactivation of the p15\(^{INK4b}\) promoter (Fig. 7, right). Thus, we conclude that the mechanism by which c-Myc impairs Ras activity in this system is Miz1-independent.

MYCBox II and HLH Domains Are Required for Inhibiting Ras Signals—To determine which regions within c-Myc are required for the impairment of Ras activity, we first tested Myc deletion mutants devoid of the N-terminal transactivation region and the C-terminal HLH region (depicted in Fig. 8A, left). The N-terminal region, harboring the conserved MYC-Box I and II regions, and the C-terminal region, spanning the Max-interacting helix, are required for Myc-mediated transactivation and transformation (21). The deficiency in transactivation activity of the Myc D1–98, Myc D106–143, and Myc D348–439 constructs in K562 cells was confirmed with the aid of a luciferase reporter that contains Myc-responsive E-boxes in a minimal promoter (not shown). The results of the luciferase assays with the p21-Luc reporter indicated that c-Myc Box II, HLH, and, to a lesser extent, c-Myc Box I were required for the inhibition of Ras-mediated activation of the p21 promoter (Fig. 8A, right). These results indicated that the regions known to be essential for c-Myc transcriptional activity are also required for antagonizing Ras. Interestingly, p21 promoter activity increased when the mutant lacking Box II and HLH domains (D106–143 and D348–439) were co-expressed with Ras (Fig. 8A). We then asked whether the Myc DNA binding region was also required. To this end we used the c-Myc-In373 mutant, impaired for DNA binding. We showed previously that this mutant was able to bind Max but was devoid of transactivation activity in K562 cells (65). The results (Fig. 8A) indicated that this mutant was unable to antagonize Ras-mediated transactivation of the p21 promoter.

Mad1 antagonizes c-Myc-mediated transactivation and transformation (21, 66). To further study whether Myc transactivation activity was required for impairment of the Ras-mediated transactivation of p21 we co-transfected c-Myc with Mad1 vector and asked whether Mad1 could abrogate the suppressive effects of c-Myc over Ras-mediated transactivation of p21. As shown in Fig. 8B, when Ras was co-transfected with c-Myc and Mad1, p21 promoter activity was significantly elevated with respect to that of cells transfected with only Ras and c-Myc. Altogether, these results indicate that those regions within Myc required for transformation and transcriptional activity are required for c-Myc-mediated repression of Ras activity on the p21 promoter.

Myc Impairs Ras-mediated Growth Inhibition in K562 Cells—Finally, we asked whether the effect of c-Myc on the regulation of p21 expression by Ras was reflected on cell growth. In other words, we asked whether c-Myc could rescue the proliferation arrest of K562 cells induced by Ras. The
enforced expression of high levels of c-Myc also inhibited clonogenicity of K562/S cells in a dose-dependent manner. As described in other models, this effect is likely due to c-Myc-induced apoptosis, as it was partly relieved by using high serum concentrations (not shown) and by enforced expression of Bcl2 (see below). To allow the study of Myc and Ras functional interaction by clonogenic assays, we first generated a K562/S subline with constitutive expression of Bcl-2 (not shown), as K562 cells do not express this antiapoptotic protein (67). We co-transfected K562/S-Bcl2 cells with Ras-G12V-T35S and c-Myc expression vectors in a ratio of 5:1 (Ras/Myc) and scored colonies after selection with hygromycin B, the resistance gene harbored in the c-Myc vector. The luciferase activity was measured over a set of p21 promoter constructs carrying mutations in several Sp1 sites as indicated in panel A (51). Data are mean values from three independent experiments. Error bars indicate S.E.

**Fig. 6.** Ras-mediated activation of p21 depends on Sp1 sites, and c-Myc impairs Ras-mediated activation of Sp1. A, schematic representation of the p21 promoter included in a -93p21-Luc construct. The binding sites of Sp1 and Miz1 are indicated (31, 32, 51). B, pCEFL-H-Ras-G12V and pCEFL-c-Myc were co-transfected using 5 μg of each plasmid. The luciferase activity was measured over a set of p21 promoter constructs carrying mutations in several Sp1 sites as indicated in panel A (51). Data are mean values from three independent experiments. Error bars indicate S.E.

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Myc Impairs Ras-mediated Growth Arrest and Activation of p21

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DISCUSSION

Even though the Ras signaling pathways that lead to cell proliferation and transformation have been studied in detail, there is little information on Ras-mediated signaling in cancer models where Ras induces growth inhibition. CML-derived K562 cells constitute one of these models.

We showed previously that H-, K-, and N-Ras oncoproteins arrest the proliferation of CML-derived K562 cells and that this arrest is, at least in part, due to Ras-mediated induction of p21 (14). In this work we have first analyzed the signaling pathways utilized by Ras to transactivate the p21 promoter. We found that this effect is mainly achieved by signaling through the Raf-MEK-ERK pathway. This conclusion is based on the following pieces of evidence (Fig. 2): (a) Ras effector mutants that only signal through Raf are able to up-regulate p21 but not those mutants signaling through PI3K or RAL-GDS. (b) p21 up-regulation is abolished by the MEK inhibitor PD98059 but not by the PI3K inhibitor LY294002; and (c) high levels of constitutively activated forms of Raf and ERK also activate the expression of p21. As anticipated from previous reports (18, 39, 40, 59, 60), the Raf-specific effector mutants (G12V-T35S and G12V-D38E) showed a decreased activity compared with Ras-G12V (Fig. 2). Thus, although other mechanisms elicited by Ras may also contribute to p21 up-regulation, they must be distinct from the so far canonical pathways activated by our mutants (through Raf, PI3K, Ral-GDS, and NORE/RASFF).

The same Ras mutants that activate the p21 promoter are also the ones that most potently inhibit the clonogenic growth of K562 (Fig. 3), arguing that the up-regulation of p21 through the Raf-ERK pathway is relevant for Ras-induced growth arrest. Consistently, actively growing K562 cells show low ERK activity levels (68), and the levels of p21 are undetectable. However, we found that Ras mutants signaling through PI3K or RAL-GDS also have antiproliferative effects in K562 (Fig. 3) even though they cannot activate p21 expression (Fig. 2). Thus, a high intensity Ras signal activates other mechanisms besides p21 up-regulation to achieve growth arrest.

K562 cells carry inactivating mutations in p53 (33, 69) and homozygous deletions of p14ARF (70, 71), p16INKa (70, 72), and p14ARF (14, 73). Therefore, the pathway Ras → p14ARF → p53 → p21, reported previously in other systems to explain Ras-mediated induction of p21, is disabled in K562. Thus, our data argue for a pathway Ras → p21 that is independent of p53, p14ARF, p15INKa, and p16INK4a. However, the expression of p21 is subject to complex regulation and can be induced by other pathways. For example, a cooperative mechanism between ERK and p38 kinases for p21 up-regulation has been described recently (74). Our results, which show that activated RAS has growth inhibitory effects in CML-derived K562 cells, are consistent with the absence of RAS mutations in CML even in the blastic terminal phase of the disease (6, 75, 76).

Several reasons led us to ask whether c-Myc modified the Ras-mediated activation of p21 expression in K562 cells. First, in addition to the absence of RAS mutation, c-Myc appears to be associated with CML progression. For example, c-Myc expression has been found to be higher in cells derived from blast crisis than from the chronic phase (77–79), and there is a positive correlation of MYC amplification or up-regulation with progression to blast crisis (27–29) or treatment failure. Thus, a study of the functional cross-talk between c-Myc and Ras could be relevant to explain MYC activation in this tumor. Second, although it has long been known that Ras and c-Myc oncogenes cooperate in the cell transformation of primary cells in vitro (36) and in vivo models (35), there is no information on Ras-Ras interaction in cancer cells where mutated Ras is antiproliferative, as K562. Third, we and others have previously described in K562 cells a c-Myc-mediated repression of p21 transcription both by antagonizing p53-dependent transactivation (33) and p53-independent mechanisms (32).

We have found that co-expression of c-Myc reduces activation of the p21 promoter by Ras-activated mutants. The effect was observed for H-, K-, and N-Ras proteins as well as for activated Raf and MEK (Figs. 4 and 5). We have not only observed an antagonistic effect of c-Myc on the effects of Ras at the level of p21 promoter activation but also at the level of Ras-induced growth impairment. c-Myc did not completely abrogate the Ras antiproliferative effect, but, as discussed above, Ras uses other mechanisms, in addition of p21 up-regulation, to impair growth. Also, under our experimental conditions it is likely that the levels of transfected c-Myc were below those of Ras. It is of note that the extent of growth rescue achieved by c-Myc in K562 is in agreement with the one observed when utilizing antisense-p21, as we have described previously (14). Taken together, our results indicate that c-Myc antagonizes the Ras-mediated inhibition of cell growth and that this is achieved, at least partially, by impairing p21 up-regulation. In agreement with this, it has been reported recently that the absence of p21 enhances the cooperation between Ras and c-Myc in rodent fibroblast transformation (80). Considering that Ras is a key regulator of p21 expression, it is conceivable that a connection

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exists between Ras and Myc functions in the upbringing of a malignant transformation in cancer cells where Ras is antiproliferative, such as K562. Indeed, herein we prove that to be the case.

Several mechanisms have been proposed to explain the cooperation between Ras and Myc in transformation, such as synergistic activation of cyclins (81–83), stabilization of the c-Myc protein (84, 85), or impairment of FoxO transcriptional repressor (86). In contrast, the mechanisms by which c-Myc antagonizes Ras-mediated growth arrest remain largely unknown. We found that activation of the p21 promoter by Ras requires Sp1/3 binding sites in the K562 model, confirming our previous results in COS cells (51). In contrast, the mechanisms by which c-Myc antagonizes Ras-mediated growth arrest remain largely unknown. We found that activation of the p21 promoter by Ras requires Sp1/3 binding sites in the K562 model, confirming our previous results in COS cells (51). We also found that c-Myc impaired Ras-mediated transcriptional activity of Sp1 rather than the binding of Sp1 to DNA (Fig. 6). Considering that ERK phosphorylates Sp1 in other systems (87–89), a possible mechanism to explain c-Myc effects would be the repression of ERK activity. However, we have demonstrated that the activity of ERK, as assessed by the phosphorylation/activation of ELK, a canonical ERK substrate, was not affected by c-Myc in this system. Further work is aimed at testing the possibility that c-Myc can induce the recruitment of co-activators to Sp1.

Also, it has been reported that, in fibroblasts and colon cancer cells, c-Myc forms complexes with Sp1/3 through its central region (amino acids 143–352) (63). Thus, it was possible that Myc could impair Ras-mediated p21 transcription by sequestering Sp1. However this possibility seems unlikely in K562 cells for the following reasons. (a) A c-Myc deletion mutant lacking 27 amino acids of the central domain (244–271) impaired Ras-mediated transcription of p21 to the same extent as wild-type c-Myc did. (b) Whereas the c-Myc N terminus, containing the c-Myc Box 1 and Box II regions, and the C-terminal Max-interacting region are not required for p21 repression in colon cancer cells (63), we found that both regions

FIG. 8. Myc BoxII and HLH domains of c-Myc are required for Ras inhibition of p21 promoter. A, a collection of c-Myc deletion mutants was constructed (see “Experimental Procedures”), and their abilities to impair Ras-mediated activation of p21 were compared on a p21-Luc construct. All of the c-Myc genes were coned in the pME18S vector except for the c-Myc In373 mutant, which was cloned in the pMLV vector. The vector (pME18S or pMLV) or each c-Myc construct (10 μg) were co-transfected with pCEFL-H-Ras-G12V (2 μg). For comparison, the effect of wild-type c-Myc in the same vector as Myc In373 is also shown (MLV-Myc). The luciferase activity observed in cells transfected with Ras and the c-Myc empty vector was set at 100%, and the other data were normalized to this value. Data are mean values from four independent experiments for In373 and six experiments for the other mutants. Error bars indicate S.E. TAD, transcriptional activation domain; NLS, nuclear localization signal; DBD, DNA-binding domain; LZ, leucine zipper. B, effect of Mad1 on c-Myc activity. pCEFL-H-Ras-G12V, pCEFL-c-Myc, and pCEFL-Mad1 were co-transfected in a proportion of 1:4:4, and activation of the p21 promoter was assayed on the p21-Luc reporter. Data are mean values from six independent experiments. Error bars indicate S.E. **, p < 0.01.
were required for the repressing effect of Myc on Ras-mediated activation of the p21 promoter. (c) Furthermore, a c-Myc insertion mutant in the DNA binding region was also unable to repress Ras activity on the p21 promoter (Fig. 8). It must be noted that the same c-Myc regions required for antagonizing Ras are also required for transformation, transactivation, and repression. Moreover, c-Myc effects could also be reversed by the co-expression of Mad1. Thus, although our results do not rule out a direct interaction between c-Myc and Sp1, it seems that the mechanism operating for the Ras-Myc interference in the K562 model does not depend on such interaction. Conversely, our data suggest that c-Myc affects Ras-mediated transactivation through the regulation of one or several c-Myc target genes that, in turn, modulate Ras activity.

It has recently been found that c-Myc represses the p21 promoter through the interaction with the zinc finger protein Miz1 (30, 31), and we have shown previously that phorbol ester-mediated up-regulation of p21 was impaired by c-Myc in a Miz1-dependent manner (32). However, a c-Myc mutant unable to bind Miz1 (c-Myc-V394D) provoked a similar impairment of the effects of Ras on p21 as wild-type c-Myc did, ruling out a role for Miz1 in the repression of p21 mediated by Ras.

Whatever the molecular mechanism involved in c-Myc-Ras interference might be, our results do the following: (a) demonstrate the importance of Ras-Raf-ERK-p21 pathway for Ras-dependent growth arrest of CML cells; and (b) suggest that the inhibition exerted by c-Myc on Ras antiproliferative activity is one of the mechanisms whereby c-Myc deregulation in CML (and probably other tumor types) leads to the progression of the disease.

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REFERENCES

1. Macara, I. G., Lounsberry, K. M., Richards, S. A., McKiernan, C., and Bar-Sagi, D. (1996) FASEB J. 10, 625–630
2. Bos, J. L. (1997) Biochim. Biophys. Acta 1333, M19–M31
3. Campbell, S. L., Khorasavi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1413
4. Kholkin, Khatchaturov, A., Rahazadeh, S., Xavier, R., Nedwodek, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. (2002) Curr. Biol. 12, 253–265
5. Kelley, G. G., Reks, S. E., Ondrako, J. M., and Smrcka, A. V. (2001) EMBO J. 20, 743–754
6. Watzinger, F., Gaiger, A., Karlic, K., Hecher, R., Pillrueh, R., and Kline, L. T. (1994) Cancer Res. 54, 3934–3938
7. Beaupre, D. M., and Kurzrock, R. (1999) J. Clin. Oncol. 17, 1071–1079
8. Calabretta, B., and Perezhod, D. (2004) Blood 103, 4010–4022
9. Liu, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. (1998) Genes Dev. 12, 3008–3019
10. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Cell 88, 593–602
11. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997) Cell. Biol. Cell. 15, 5586–5611
12. Malumbres, M., and Pellicer, A. (1998) Front. Biosci. 3, 887–891
13. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berget, S., and Perry, S. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7992–7997
14. Grosveld, F., Ailhaud, G., and Wassef, M. (1990) EMBO J. 9, 10611–10618
15. Li, Z., Van Calcar, S., Qu, C., Cavenee, W. K., Zhang, M. Q., and Ren, B. (2003) Mol. Cell. Biol. 23, 7041–7052
16. Sewing, A., Wiesner, B., Lloyd, A. C., and Land, H. (1997) Mol. Cell. Biol. 17, 8588–5097
17. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997) Cell. Biol. Cell. 15, 5586–5611
18. Hamad, N. M., Elizondo, J. J., Javier Vasquez, A. B., Bai, W., Rich, J. N., Abraham, R. T., Der, C. J., and Counter, C. M. (2002) Genes Dev. 16, 2045–2057
19. Lutz, W., Leon, J., and Eilers, M. (2002) Biochim. Biophys. Acta 1602, 61–71
20. Eisenman, R. N. (2001) Genes Dev. 15, 2023–2030
21. Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000) Annu. Rev. Cell Dev. Biol. 16, 653–699
22. Haggerty, T. J., Zeller, K. I., Osthuz, R. C., Wonsey, D. R., and Dang, C. V. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5313–5318
23. Oster, S. K., Ho, C. S., Soucie, E. L., and Penn, L. Z. (2002) Adv. Cancer Res. 84, 81–154
24. Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) Cancer Genet. Cytogenet. 81, 329–356
25. Beck, Z., Baici, A., Kovacs, E., Kiss, J., Kiss, A., Balogh, E., Telek, B., Toth, F. D., Andirko, I., Olah, E., Udvardy, M., and Rak, K. (1998) Leuk. Lymphoma 30, 293–306
26. Jennings, B. A., and Mills, K. I. (1998) J. Biol. Chem. 273, 35115–35120
27. Beuger, V., Eilers, M., Leon, J., and Larsson, L. G. (2003) Oncogene 22, 351–360
28. Celis, E., Delgado, M. D., Gutierrez, P., Richard, C., Muller, D., Eilers, M., Ehinger, M., Gullberg, U., and Leon, J. (2000) Oncogene 19, 2194–2204
29. Compere, S. J., Baldacci, P., Sharpe, A. H., Thompson, T., Land, H., and Jaenisch, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2224–2228
30. Stone, J., De Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H., and Wigler, M. H. (1995) Cell 80, 531–541
31. Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) Cancer Genet. Cytogenet. 81, 329–356
32. Reynolds, W., Cetinkaya, C., Munoz-Alonso, M. J., von der Lehr, N., Bahram, F., Beuger, V., Eilers, M., Leon, J., and Larsson, L. G. (2003) Oncogene 22, 351–360
33. Celis, E., Delgado, M. D., Gutierrez, P., Richard, C., Muller, D., Eilers, M., Ehinger, M., Gullberg, U., and Leon, J. (2000) Oncogene 19, 2194–2204
34. Compere, S. J., Baldacci, P., Sharpe, A. H., Thompson, T., Land, H., and Jaenisch, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2224–2228
35. Stone, J., De Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H., and Lee, W. (1987) Mol. Cell. Biol. 7, 1697–1709
36. Takebe, Y., Seiki, M., Fujisawa, J., Huy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) Mol. Cell. Biol. 8, 466–472
Myc Impairs Ras-mediated Growth Arrest and Activation of p21

46. Shiio, Y., Yamamoto, T., and Yamaguchi, N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5206–5210
47. Hagen, G., Dening, J., Preiss, A., Beato, M., and Suske, G. (1995) J. Biol. Chem. 270, 24989–24994
48. Jannecke, R., and Hunter, T. (1997) EMBO J. 16, 1620–1627
49. Botz, J., Zerfass-Thome, K., Spikovsky, D., Delius, H., Vogt, B., Eilers, M., Hatzigeorgiou, A., and Jansen-Durr, P. (1996) Mol. Cell. Biol. 16, 3401–3409
50. Hurlin, P. J., Queva, C., and Eisenman, R. N. (1997) Genes Dev. 11, 44–58
51. Kivinen, L., Trubari, M., Haapajarvi, T., Datto, M. B., Wang, X. F., and Laiho, M. (1999) Oncogene 18, 6252–6261
52. Sadowski, I., and Ptashne, M. (1989) Nucleic Acids Res. 17, 7539
53. Malumbres, M., Perez De Castro, I., Hernandez, M. I., Jimenez, M., Cuenca, J., and Pellicer, A. (2000) Mol. Cell. Biol. 20, 2915–2925
54. Perez de Castro, I. P., Malumbres, M., Santos, J., Pellicer, A., and Fernandez-Piqueras, J. (1997) Cancer Res. 57, 6252–6261
55. Gartel, A. L., Goufman, E., Tevosian, S. G., Shih, H., Yee, A. S., and Tyner, A. L. (1998) Oncogene 17, 3483–3498
56. Goufman, E., and Gartel, A. L. (1998) Leuk. Res. 22, 1003–1007
57. Aneziris, I., Matallanas, D., Berciano, M. T., Sanz-Moreno, V., Calvo, F., Munoz, M. T., Egea, G., Lafarga, M., and Crespo, P. (2004) Mol. Cell. Biol. 24, 1516–1530
58. Chio, V. K., Rivova, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., II, Cox, A. D., and Philips, M. R. (2002) Nat. Cell Biol. 4, 343–350
59. Missero, C., Pirro, M. T., and Di Lauro, R. (2000) Mol. Cell. Biol. 20, 2783–2793
60. Verheijen, M. H., Wohluim, R. M., Defize, L. H., den Hertog, J., and Bos, J. L. (1999) Oncogene 18, 4435–4439
61. Deleted in proof
62. Aneziris, I., Matallanas, D., Berciano, M. T., Sanz-Moreno, V., Calvo, F., Munoz, M. T., Egea, G., Lafarga, M., and Crespo, P. (2004) Mol. Cell. Biol. 24, 1516–1530
63. Garicochea, B., Giorgi, R., Odone, V. F., Dorhia-Llacra, P. E., and Bendit, I. (1998) Leuk. Res. 22, 1003–1007
64. Nowicki, M. O., Pawlowski, P., Fischer, T., Hess, G., Pawlowski, T., and Skorski, T. (2003) Oncogene 22, 3952–3963
65. Preier, H. D. (1990) Blood 75, 1587–1588
66. Wang, Z. Q., Yin, M. Y., Xie, X. X., Yang, P. M., Sato, H., Muyers, G., Rissowal, C., and Preier, H. D. (1999) Eur. J. Cancer 26, 694–698
67. Carnero, A., and Beach, D. H. (2004) Oncogene
68. Haas, K., Johannes, C., Geisen, C., Schmidt, T., Karsunky, H., Blass-Kampmann, S., Ohe, G., and Moroy, T. (1997) Oncogene 15, 2615–2623
69. Leone, G., DeGregori, J., Sears, R., Jakai, L., and Nevins, J. R. (1997) Nature 387, 422–426
70. Born, T. L., Frost, J. A., Schonthal, A., Prendergast, G. C., and Feramisco, J. R. (1994) Mol. Cell. Biol. 14, 5710–5718
71. Sears, R., Leone, G., DeGregori, J., and Nevins, J. R. (1999) Mol. Cell 3, 169–179
72. Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K., and Nevins, J. R. (2000) Genes Dev. 14, 2501–2514
73. Bouchard, C., Marquardt, J., Bras, A., Medema, R. H., and Eilers, M. (2004) EMBO J. 23, 2830–2840
74. Milani-Mongiat, J., Pouysegur, J., and Pages, G. (2002) J. Biol. Chem. 277, 20631–20639
75. Liu, A., Prenger, M. S., Norton, D. D., Mei, L., Kusiak, J. W., and Bai, G. (2001) J. Biol. Chem. 276, 45372–45379
76. Chupreta, S., Du, M., Todisco, A., and Merchant, J. L. (2000) Am. J. Physiol. 278, C697–C708
77. Uti, L., Liu, J., Gish, G., Mbamalu, G., Bowett, D., Pelici, P. G., Arlinghaus, R., and Pawson, T. (1994) EMBO J. 13, 764–773
78. Cortez, D., Stius, G., Pierce, J. H., and Pendergast, A. M. (1996) Oncogene 13, 2589–2594
79. Raitano, A. B., Halpern, J. R., Hambuch, T. M., and Sawyer, C. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11746–11750
80. Otsuki, T., Clark, H. M., Wellmann, A., Jaffe, E. S., and Raffeld, M. (1995) Cancer Res. 55, 1436–1440