Targeting of Miz-1 Is Essential for Myc-mediated Apoptosis*

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The c-Myc oncogene plays a central role in human cancer via its ability to either activate or repress the transcription of essential downstream targets. For many of the repressed target genes, downregulation by c-Myc relies on its ability to bind and inactivate the transcription factor Miz-1. Although Miz-1 inactivation is suspected to be essential for at least some of the biological activities of c-Myc, it has been difficult to demonstrate this requirement experimentally. Using a combination of short hairpin RNA-mediated knockdown and a previously characterized mutant of c-Myc that is defective for Miz-1 inactivation, we examined whether this inactivation is critical for three of the most central biological functions of c-Myc, cell cycle progression, transformation, and apoptosis. The results of this analysis demonstrated that in the in vitro assays utilized here, Miz-1 inactivation is dispensable for c-Myc-induced cell cycle progression and transformation. In marked contrast, the ability of c-Myc to induce apoptosis in primary diploid human fibroblasts in response to growth factor withdrawal is entirely dependent on its ability to inactivate Miz-1. These data have a significant impact on our understanding of the biochemical mechanisms dictating how c-Myc mediates opposing biological functions, such as transformation and apoptosis, and demonstrate the first requirement for Miz-1 inactivation in any of the biological functions of c-Myc.

The c-myc oncogene is among the most commonly overexpressed genes in human cancer (1, 2). Its protein product, c-Myc, encodes a sequence-specific transcription factor that functions largely by regulating the expression of downstream target genes (reviewed in Ref. 3). Like many transcriptional regulators, c-Myc has the ability to activate some targets while repressing others (4). Transcriptional activation by c-Myc involves the recruitment of histone acetyltransferases and other coactivators (5–10). These enzymes modify the chromatin surrounding c-Myc target gene loci, thereby facilitating increased transcription. In contrast, transcriptional repression by c-Myc appears to involve multiple, distinct mechanisms (11–15). At the biochemical level, the best characterized of these mechanisms depends on c-Myc binding and inhibiting the activity of the transcriptional activator Miz-1 (Myc-interacting zinc-finger protein-1) (16–19). In the absence of c-Myc, Miz-1 binds to specific genes and activates their transcription (4). When overexpressed, c-Myc binds directly to Miz-1 and blocks this transcription (18–21). Mechanistically, c-Myc binding to Miz-1 at target promoters prevents recruitment of cofactor proteins like p300 (18). In addition, c-Myc has been shown to recruit the DNA methyltransferase Dnmt3a to Miz-1 targets, resulting in the formation of a ternary complex composed of c-Myc, Miz-1, and Dnmt3a (11). Following recruitment, Dnmt3a methylates surrounding DNA, yielding a closed chromatin state. Miz-1 regulates the expression of several hundred genes and initial studies suggest that many of these can be repressed by c-Myc (22, 23). To date, detailed studies have been conducted primarily on Miz-1 targets of the cyclin-dependent kinase inhibitor class such as p15INK4b (CDKN2B) and p21CIP1 (CDKN1A) (12, 17–19, 24–27). The demonstration that c-Myc represses these cell cycle inhibitors has raised the hypothesis that repression of either these or other Miz-1 targets is essential for c-Myc function.

The major biological consequences of c-Myc overexpression include enhanced cell cycle progression, malignant transformation, and increased apoptosis, depending on the cellular context (28–31). The current study was designed to assess which of these functions depends on the ability of c-Myc to bind and inactivate Miz-1. Using a panel of in vitro assays, we find that repression of the Miz-1 pathway is dispensable for the ability of c-Myc to induce both cell cycle progression and transformation. In contrast, the ability to bind and inactivate Miz-1 is required for c-Myc’s induction of apoptosis in growth-factor-deprived primary human fibroblasts. These data provide the first evidence that any of the major biological activities of c-Myc require its ability to repress the Miz-1 pathway and suggest that it may be possible to distinguish the genetic pathways linking c-Myc to transformation from those linking c-Myc to apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids—The V394D mutation was introduced into the retroviral expression vector pMSCV c-Myc(puro) by site-directed mutagenesis (Stratagene) following manufacturer’s suggestion. Luciferase and Miz-1 shRNA3 constructs were cloned into the pSuper.retro.puro plasmid (OligoEngine). Target sequences of shRNA constructs are luciferase, 5′-CTTACGCTGAGTACTTCGA, and Miz-1, 5′-AAGCGGAAGCGTTATCAGGA. The Migr1 Myc/ER (32) and cytomegalovirus-driven c-Myc expression vectors (5) have been previously described.

Cell Culture and Transient Transfections—All cell lines were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 2 μM glutamine, 100 μg/ml penicillin (Invitrogen), and 100 μg/ml streptomycin sulfate (Invitrogen). Lipofectamine 2000 (Invitrogen) and OPTI-MEM I reduced serum media (Invitrogen) were used in transient transfections following manufacturer’s protocol. For experiments involving DNA damage, cells were treated with 50 J/m2 UV light using Stratalinker 1800 (Stratagene).

Retroviral Infections—Retroviral vectors and the SVψ-A-MLV helper plasmid (33) were cotransfected into the 293T packaging cell line. Virus-containing supernatant was collected, and four sequential viral infections were performed on target cells 24–48 h post-transfection in

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3 The abbreviations used are: shRNA, short hairpin RNA; ER, estrogen receptor; CKI, cyclin-dependent kinase inhibitor.
Apoptosis by c-Myc Requires Miz-1

the presence of 8 μg/ml Polybrene. Infected cell populations were subsequently enriched by either puromycin selection or sorting for green fluorescent protein-positive cells.

Soft Agar Assays—Soft agar assays were performed as described previously (32). Briefly, 2 × 10⁵ Rat1a c-Myc/ER-, Rat1a c-Myc WT-, or Rat1a c-Myc V394D-expressing cells were seeded in Dulbecco’s modified Eagle’s medium containing 0.3% agarose above a layer of Dulbecco’s modified Eagle’s medium containing 0.6% agarose in 6-well plates. After 24 h, 2 ml of Dulbecco’s modified Eagle’s medium with or without 200 nM 4-hydroxytamoxifen was added to each well. Colony formation was qualitatively assayed by phase-contrast light microscopy on day 10. To quantitate colony formation, plates were scanned on day 12 and colonies counted.

Apoptosis Assays—Following puromycin selection, primary human fibroblasts were seeded at 5 × 10⁵ cells/10-cm dish. The following day, cells were washed thoroughly with phosphate-buffered saline and then maintained in 0.2% fetal bovine serum containing Dulbecco’s modified Eagle’s medium for 24 h. Serum-starved cells were stained using an annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Pharmingen).

Cell Cycle Analysis—For proliferation assays, cells were seeded at low density (2 × 10⁵) in 6-cm dishes and counted on subsequent days. Cell cycle profiles were determined by staining fixed cells with propidium iodide (Sigma) following the manufacturer’s suggestion and subsequent flow cytometry ( Coulter Epics XL-MCL).

Immunoblotting and Coimmunoprecipitation Analysis—Whole cell lysates were prepared using a Nonidet P-40-based buffer (5). Miz-1 (Santa Cruz), c-Myc (Santa Cruz), Max (Santa Cruz), p21CIP1 (Santa Cruz), p15INK4B (Santa Cruz), p53 (Santa Cruz), β-tubulin (Sigma), and actin (Santa Cruz) antibodies used for Western blotting and coimmunoprecipitation were obtained from commercial sources. For protein-protein interaction studies, ~750 μg of whole cell lysates were immunoprecipitated with 5 μg of antibody under high salt conditions (250 mM NaCl). Western blots were performed as previously reported (5).

Real Time Reverse Transcription-PCR Analysis—Total RNA was isolated from cells using RNaseasy (Qiagen). SuperScript II Rhase H-reverse transcriptase (Invitrogen) was used to produce cDNA according to manufacturer’s protocol. Quantitative reverse transcription PCR was performed using the 7000 sequence detection system (ABI Prism) and SYBR GREEN PCR Master Mix (Applied Biosystems) amplifying products of ~50 bp. Primer sequences are as follows: actin (5′-CGTACGCC-ATCCAGGCTGTG, 5′-CCAGTGTTACGACCAGAGGC), Miz-1 (5′-AAAGCTTTCTAGGAGGG, 5′-CCACCCCACTCTATTCCAG), vimentin (5′-GAGCCACCTCCTAGTCTTCA, 5′-CGTGCCAGAGAGCATTTC), p15 (5′-ATGCTCAAGGCCCTGATA, 5′-CATCATGACCTGATCCAG), and p21 (5′-GATCCGTTGAGTGTCGACC, 5′-AGCAGCGCCACTTGGCTCC). Analysis of each sample was performed in triplicate and normalized to actin mRNA levels.

RESULTS

As discussed above, c-Myc represses the transcription of genes activated by Miz-1. However, this well-characterized biochemical function of c-Myc has not yet been linked to any of the biological effects of c-Myc. Thus, studies were conducted to examine the requirement for inactivating Miz-1 in the ability of c-Myc to induce cell cycle progression, transformation, or apoptosis.

If efficient inactivation of Miz-1 is essential for c-Myc-induced transformation, then decreasing Miz-1 function by directly targeting it with shRNA should cooperate with c-Myc by increasing transforming potential. As an initial test of whether targeting the Miz-1 pathway increases c-Myc induced transformation, soft agar assays were performed. This assay measures anchorage-independent growth, and the results obtained correlate closely with the ability of cells to form tumors in vivo. The assays included a conditional allele of c-Myc (c-Myc/ER), in which the full-length c-Myc protein is fused with the hormone-binding domain of the estrogen receptor (31, 32, 34, 35). Although c-Myc/ER is constitutively expressed, it is active only upon exposure to the estrogen analog, 4-hydroxytamoxifen. Stable pools of Rat1a fibroblasts expressing c-Myc/ER were generated, and the expression of c-Myc/ER was documented over the course of the assay (Fig. 1A). These cells were subsequently infected with retroviruses encoding Miz-1 shRNA or a luciferase shRNA as a negative control. Although antibodies detecting the endogenous rat Miz-1 protein are not currently available, shRNA infection resulted in a significant decrease in Miz-1 mRNA levels, as assessed by quantitative reverse transcription-PCR (Fig. 1B). As expected, activation of c-Myc/ER resulted in soft agar colony formation (Fig. 1, C and D). As discussed above, if inactivation of Miz-1 is an essential event during c-Myc-induced transformation, then the depletion of Miz-1 by shRNA would be predicted to synergize with c-Myc in transformation assays. However, knock down of Miz-1 failed to increase soft agar growth, suggesting that inactivation of Miz-1 may not be an essential function of c-Myc in transformation.

Among the most well characterized effects of the binding of c-Myc to Miz-1 is the transcriptional repression of the cyclin-dependent kinase inhibitors (CKIs) p15INK4b and p21CIP1. The targeting of Miz-1 by c-Myc blocks CKI transcription. Should CKI induction be compromised a priori in Rat1a cells by mutation, this might explain why depletion of Miz-1 failed to augment transformation. To examine whether transcriptional regulation of the CKIs is intact in Rat1a fibroblasts, cells were exposed to UV light and analyzed for p21CIP1 and p15INK4B protein and mRNA induction. As a control, induction of the tumor suppressor p53 by DNA damage was assessed in parallel. Although undetectable in untreated cells, p53 was up-regulated following UV irradiation (Fig. 2A). The induction of p21CIP1, which is regulated by p53, was also induced by DNA damage, at both the protein (Fig. 2A) and mRNA (Fig. 2B) levels. Similarly, p15INK4b mRNA and protein were induced in response to DNA damage (Fig. 2, A and C), consistent with previous observations (24). These data suggest that the pathways regulating these CKIs are intact in Rat1a cells. Transcription of the gene encoding the intermediate filament protein vimentin was not responsive to DNA damage, negating the possibility of a nonspecific, global increase in transcription (Fig. 2D). These data suggest that the lack of synergy between c-Myc activation and Miz-1 depletion does not result from pre-existing mutations in the Miz-1 targets p15INK4b and p21CIP1.

As c-Myc may be capable of complete inactivation of Miz-1, shRNA-mediated depletion of Miz-1 might not contribute additional transformation potential. To more rigorously assess the role of Miz-1 inactivation in c-Myc-mediated transformation, a parallel system was generated utilizing a previously characterized mutant of c-Myc that is defective for Miz-1 interaction. As demonstrated in a number of studies, this mutant, V394D, lies within the helix-loop-helix domain and selectively disrupts Miz-1 binding without affecting dimerization with Max (11, 19, 24, 36–40). In addition, the V394D mutant has been shown to be defective for transcriptional repression of c-Myc targets while retaining the ability to activate the transcription of others. As such, this mutant has been widely used to distinguish between Miz-1-dependent repression functions and other functions of c-Myc. Rat1a cells were therefore infected
with retroviruses encoding wild-type c-Myc or the V394D mutant. To confirm that the V394D point mutation disrupted the interaction of c-Myc with Miz-1 as previously reported, coimmunoprecipitation assays were performed. Following transient transfection of c-Myc or Miz-1 expression plasmids, whole cell lysates were examined for c-Myc expression. To document protein expression, cells in the standard culture were harvested on subsequent days, and whole cell lysates were examined for c-Myc expression. The fold change of Miz-1 mRNA transcript levels was analyzed by quantitative reverse transcription-PCR throughout the course of the soft agar experiment. Cells seeded in soft agar were overlaid with media containing either ethanol, as a vehicle control, or 4-hydroxytamoxifen (4-OHT) to activate c-Myc/ER on day 1. Mean colony count from triplicate assays ± S.D. is shown.

To directly assess the requirement for Miz-1 interaction in the ability of c-Myc to transform cells, wild-type c-Myc and c-Myc V394D-expressing Rat1a cells were assayed for their ability to induce soft agar colony formation. This analysis revealed that colony growth was similarly induced by expression of either wild-type c-Myc or c-Myc V394D (Fig. 3E). Anchorage-independent growth was dependent on c-Myc expression, as only vestigial colonies formed in vector-infected cells. Quantitation revealed equivalent colony formation between cells expressing wild-type c-Myc and those expressing the V394D mutant, suggesting that binding and inactivation of Miz-1 are dispensable for transformation in vitro.

As both the shRNA and V394D studies suggest that Miz-1 inactivation may not be essential for the transforming function of c-Myc in vitro, other functions that might depend on repression of Miz-1 were explored. Previous reports have demonstrated that ectopic expression of wild-type c-Myc can genetically rescue the cell cycle defects exhibited by the c-myc−/− Rat1 cell line HO15.19 (41). We therefore compared the ability of wild-type and V394D c-Myc proteins to achieve this rescue. After documenting equivalent protein expression for wild-type and V394D c-Myc proteins by Western blot (Fig. 4A), proliferation rates and
cell cycle profiles were examined. Despite the inability of c-Myc V394D to bind and inactivate Miz-1, the mutant protein rescued the slow growth phenotype of HO15.19 cells to the same extent as wild-type c-Myc (Fig. 4B). To examine changes in specific stages of the cell cycle, cells were stained with propidium iodide and analyzed by flow cytometry. Cells expressing either wild-type c-Myc or c-Myc V394D displayed an increase in the S-phase population and a significant decrease in the G0/G1 population (Fig. 4, C and D), correlating with the increase in
proliferation. The virtually identical effects of wild-type c-Myc and c-Myc V394D in c-myc−/− HO 15.19 cells suggest that inactivation of Miz-1 does not significantly contribute to the ability of c-Myc to regulate cell cycle progression.

These data suggest that there may not be a strict requirement for targeting of the Miz-1 pathway in either cell cycle progression or transformation. Therefore, a role for this interaction in c-Myc-induced apoptosis was examined. The primary diploid human fibroblast strain IMR90 was engineered to constitutively express either wild-type or the V394D mutant of c-Myc, as confirmed by Western blotting (Fig. 5A). Withdrawal of serum growth factors from cells expressing wild-type c-Myc resulted in a significant increase in apoptosis, relative to vector-infected cells (Fig. 5B). Remarkably, the V394D mutant of c-Myc was completely defective for apoptosis induction. To confirm that the requirement for Miz-1 targeting in c-Myc-induced apoptosis is not a unique feature of the IMR90 fibroblasts, the studies were repeated in another human diploid fibroblast strain, 2091. As expected, withdrawal of serum growth factors from 2091 cells expressing wild-type c-Myc resulted in significant apoptosis (Fig. 5C). Consistent with the data obtained in IMR90 cells, 2091 cells expressing the Miz-1 interaction mutant V394D were defective for apoptosis induction.

Considered together, these data suggest that repression of Miz-1 activity is not essential for the induction of c-Myc of cell cycle progression and transformation but is required for c-Myc-induced apoptosis. The observations represent the first of the biological activities of c-Myc to be linked to Miz-1 inactivation.

**DISCUSSION**

An array of recent studies have demonstrated that c-Myc can repress transcription by binding and inactivating the transcriptional activator Miz-1 (11, 18–20, 24, 25). To accomplish this, c-Myc displaces coactivators from Miz-1 and recruits the DNA methyltransferase DNMT3a to genes where Miz-1 would otherwise activate transcription (11, 18). Although these biochemical events are well established, what has remained unresolved is the role that Miz-1 inactivation plays in the diverse biological activities of c-Myc.

In this study, we examined the requirement for Miz-1 inactivation in the c-Myc functions of transformation, cell cycle progression, and apoptosis. For c-Myc-induced cell cycle progression, we demonstrated that a mutant selectively defective for inactivating Miz-1 (V394D) is competent for rescuing cell cycle defects in c-myc−/− cells. These findings are consistent with previous studies showing that knockdown of Miz-1 does not affect cell growth (22) and with reports that rat fibroblasts overexpressing wild-type c-Myc and V394D have comparable proliferation rates (24). Combined, these data suggest that at least under some conditions, the ability of c-Myc to induce cell cycle progression can be independent of Miz-1 inactivation.

This study, like a number of others (11, 19, 24, 36–40), has relied on
the use of the V394D mutant to selectively block the ability of c-Myc to bind and inactivate Miz-1. The assumption that this mutant is defective for only this function of c-Myc is based on a number of observations. First, the V394D mutant is indeed defective for Miz-1 interaction. Second, V394D is defective at repressing the transcription of Miz-1-activated genes. Third, the V394D mutation fails to inhibit the dimerization of c-Myc with its obligate partner Max. Dimerization with Max is the only known biochemical function of the helix-loop-helix domain in which V394 resides. Fourth, the V394D mutant is competent for the transcriptional activation of c-Myc target genes. Fifth, as shown here, the biological functions of c-Myc that depend on transcriptional activation (cell cycle progression and transformation) appear to be intact in the V394D mutant. Considered together, these data suggest that the defect in the V394D mutant is highly selective for the Miz-1 repression pathway. More formal proof that Miz-1 inactivation is the only pathway defective in the V394D mutant will require generation of a compensating mutation in Miz-1 that restores its binding to the V394D version of c-Myc.

A number of studies have pointed out that the repressive activity of c-Myc correlates with its ability to transform cells (42–44). In contrast, our examination of c-Myc’s repression of Miz-1 suggests that this specific repression pathway is not essential for cellular transformation by c-Myc. However, it remains likely that the ability of c-Myc to repress transcription through one of several other pathways is indeed critical for transformation (12–15).

Like many potent oncopgenes, c-Myc induces apoptosis when expressed in the appropriate cellular context, presumably as a mechanism of self-regulation. For example, in the absence of growth factors, fibroblasts overexpressing c-Myc undergo programmed cell death (31). We therefore examined whether the ability of c-Myc to inactivate Miz-1 is important for apoptosis induction. Remarkably, our results show that unlike cell cycle progression and transformation, Miz-1 inactivation is an essential event during c-Myc-induced apoptosis in primary human fibroblasts. These data conflict with a previous study that found no significant difference between wild-type c-Myc and the V394D mutant in apoptotic induction in immortalized rat fibroblasts (24). As the current study was conducted in primary diploid human fibroblasts, it remains possible that known mutations in the Arf pathway in the rat cell line used previously rendered the requirement for Miz-1 inactivation unnecessary (24). What remains more difficult to explain is why a defect in apoptosis induction by the V394D mutant fails to affect transformation potential. Malignant transformation by c-Myc is thought to represent a balance between cells that are overtly transformed and those that die by apoptosis (45). If apoptosis is compromised in the V394D mutant, one might expect to observe an increase in transformation potential. Although such an increase was not observed here, the in vitro transformation assay utilized may lack the costimulatory signals for apoptosis induction that exist in vivo, e.g. hypoxia and growth factor depletion. Determining whether there is an in vivo requirement for Miz-1 inactivation in transformation will ultimately require the establishment of a mouse model comparing the activity of wild-type c-Myc to V394D.

Future studies will be aimed at determining which of the targets of Miz-1 must be inactivated during c-Myc-induced apoptosis. Initial studies focusing on p15INK4B and p21CIP1 failed to find any distinction in repression by wild-type c-Myc and V394D (data not shown). Similarly, proapoptotic targets of p53 such as NOXA, PUMA, and BAX are not differentially regulated in cells expressing c-Myc and V394D. Therefore, it appears likely that the essential Miz-1 target whose repression is necessary for c-Myc-induced apoptosis is among the many other genes activated by Miz-1. In fact, recent microarray analysis has revealed that a number of anti-apoptotic genes are activated by Miz-1 (22, 23). These include BCL2, BCL2L1, MCL-1, and apoptosis inhibitor immediate early response 3 (IERS). BCL2 represents a promising target as it has previously been identified as a c-Myc-repressed target (46, 47).

Antagonism of Miz-1-mediated transcription has been postulated to play an important role in the biological activities of c-Myc. However, to date there has been no experimental data to support such a role. The demonstration here that Miz-1 inhibition is essential for c-Myc-mediated apoptosis is the first documentation of a role for this pathway in the biological function of c-Myc. This observation may advance the search for mechanisms dictating whether c-Myc activation induces transformation versus apoptosis. The ability to selectively reactivate the apoptotic pathway in human tumors transformed by c-Myc would be of significant clinical value.

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