Degrasyn Activates Proteasomal-Dependent Degradation of c-Myc

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Abstract
c-Myc is a highly unstable transcription factor whose deregulation and increased expression are associated with cancer. Degrasyn, a small synthetic molecule, induces rapid degradation of c-Myc protein in MM-1 multiple myeloma and other tumor cell lines. Destruction of c-Myc by degrasyn requires the presence of a region of c-Myc between amino acid residues 316 and 378 that has not previously been associated with c-Myc stability. Degrasyn-induced degradation of c-Myc depends on proteasomes but is independent of the degron regions previously shown to be important for ubiquitin-mediated targeting and proteasomal destruction of the protein. Degrasyn-dependent c-Myc proteolysis is not mediated by any previously identified c-Myc regulatory mechanism, does not require new protein synthesis, and does not depend on the nuclear localization of c-Myc. Degrasyn reduced c-Myc levels in A375 melanoma cells and in A375 tumors in nude mice, and this activity correlated with tumor growth inhibition. Together, these results suggest that degrasyn reduces the stability of c-Myc in vitro and in vivo through a unique signaling process that uses c-Myc domains not previously associated with c-Myc regulation. [Cancer Res 2007;67(8):3912–8]

Introduction
c-Myc is a central regulator of key cellular processes including differentiation, G1-S phase transition, proliferation, maintenance of cell size, redox state, genomic integrity, and apoptosis (1–3). Current estimates suggest that c-Myc influences 5% to 15% of the human genome (4) and is finely regulated owing to its central role in cell regulation (5, 6). Loss of control of c-Myc expression and stability occurs in many human cancers (7, 8) and is associated with highly aggressive, poorly differentiated tumors with poor patient prognosis. Ubiquitin-mediated proteolysis (9), a specific multistep process that results in the target protein being rapidly destroyed by the 20S proteasome, plays an important role in c-Myc degradation and is responsible for its short half-life (10–16).

c-Myc protein levels are regulated by a NH2-terminal "degron" that signals c-Myc ubiquitination and a COOH-terminal "stabilon" that stabilizes c-Myc by enabling it to associate with the POZ domain protein Miz or to be sequestered into a stabilizing subnuclear compartment (12, 17). The degron, located within the first 147 residues of the protein, spans the transactivation domain and includes two highly conserved sequences known as myc box I (MB I) and myc box II (MB II; refs. 12, 13). Located within MB I are two key amino acid residues (Thr38 and Ser62) whose pattern of phosphorylation determines c-Myc stability. c-MycS, a naturally occurring mutant of c-Myc, has a half-life similar to that of the full-length c-Myc despite missing the first 100 amino acids (18), suggesting that regions of c-Myc outside the degron are also important in its regulation. The D element (amino acids 190–210), a naturally occurring mutant of c-Myc, has a half-life similar to that of the full-length c-Myc despite missing the first 100 amino acids (18), suggesting that regions of c-Myc outside the degron are also important in its regulation. The D element (amino acids 190–210), which overlaps MB II, and the adjacent PEST sequence (amino acids 226–270) are necessary for rapid c-Myc proteolyis at a step after ubiquitination (19, 20). A second F-box protein (Skp2) along with its associated E3 ligase SCF complex (Skp/Cul1/F-box) interact with c-Myc and mediate the destabilization of the protein (21, 22). Skp2-binding sites have been identified between residues 129 to 147 and 379 to 418 of c-Myc. Under normal growth conditions, c-Jun NH2-terminal kinase (JNK) associates with c-Myc in a region between amino acids 127 and 189 and mediates its ubiquitination and degradation (14). The Cdc42/Rac GTPase-controlled kinase PAK2 phosphorylates c-Myc at Thr358, Ser373, and Thr400, reducing its interaction with Max and preventing c-Myc-mediated transcription while increasing its degradation through a process that remains elusive (23, 24).

The synthetic small molecule degrasyn induces rapid downregulation of c-Myc in several tumor cell types, including MM-1 multiple myeloma, HeLa cervical carcinoma, and A375 melanoma. Degrasyn was derived from a small chemical library screened for its ability to inhibit cytokine-stimulated signal transducer and activator of transcription 3 (Stat3) activation in a cell-based assay. On the basis of previous reports of a link between Jak/Stat inhibition and suppression of c-Myc (25, 26), additional compounds were synthesized and screened for their ability to directly reduce c-Myc levels in the absence of Stat3 activation. After extensive structure-activity-studies, degrasyn emerged as a potent and rapid regulator of c-Myc protein levels in multiple tumors. The NH2-terminal degron of c-Myc has been shown to be important for proteasomal-dependent degradation of c-Myc. However, there is little evidence that the central region of c-Myc serves as a major target recognition site for proteasomal-dependent degradation of c-Myc that is independent of the degron. In this study, we show that degrasyn induces the proteasomal-dependent degradation of c-Myc by targeting a region within amino acid residues 316 to 335 and 356 to 378, neither of which had previously been shown important for c-Myc degradation. This mechanism is unique, as it is not inhibited by deletion of c-Myc domains essential for ubiquitin-mediated degradation. These observations suggest that degrasyn induces degradation of c-Myc through a novel signaling mechanism that uses unique regions of c-Myc for activity.

Materials and Methods

Materials. The following materials and reagents were obtained from commercial sources: RPMI 1640 and DMEM/F12 (Cambrex, Walkersville,
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MD) and antibodies to c-Myc (Cell Signaling Technology, Danvers, MA), actin (Sigma-Aldrich, St. Louis, MO), and hemagglutinin (HA; Roche Applied Science, Indianapolis, IN). Degrasyn (Fig. 1A) was synthesized and purified at the M. D. Anderson Cancer Center.

Cell lines. MM-1 multiple myeloma cells were kindly provided by Dr. Steven Rosen (Northwestern University). A375 melanoma cells were obtained from Dr. Elizabeth Grimm (Department of Experimental Radiation Oncology at the M. D. Anderson Cancer Center). HeLa cervical carcinoma cells were provided by Dr. Mein-Chie Hung (Department of Molecular Oncology, M. D. Anderson Cancer Center). All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Transfection. HeLa cells (5 × 10^6) were transfected with 0.5 to 2 μg of HA-tagged wild-type c-Myc cDNA or cDNA of c-Myc deletion mutants (kindly provided by Dr. William P. Tansey, Cold Spring Harbor Laboratory) via the lipofection delivery system SN2 (kindly provided by Dr. Mein-Chie Hung), and the transfected cells were treated 24 h later with buffer or with 5 μmol/L degrasyn for 2 h.

Immunoblotting. Protein extracts from cell lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and blocked for 1 h at room temperature in 5% dry milk/TBS/0.1% Tween. Incubation with primary antibody (diluted in 5% bovine serum albumin/TBS/0.1% Tween) overnight at 4°C was followed by a 1-h incubation with horseradish peroxidase–labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 5% dry milk/TBS/0.1% Tween. Membranes were then developed with enhanced chemiluminescence substrate (Amersham Biosciences, Piscataway, NJ).

Plasmid DNA manipulations. The mammalian expression vector for HA epitope-tagged c-Myc was obtained by cloning the wild-type c-Myc coding sequence into pcG3 (kindly provided by Dr. William P. Tansey). Myc mutants deleted at amino acids 316 to 336, 337 to 357, 358 to 378 or with coding sequence into pcG3 (kindly provided by Dr. William P. Tansey, Cold Spring Harbor Laboratory) via the lipofection delivery system SN2 (kindly provided by Dr. Mein-Chie Hung), and the transfected cells were treated 24 h later with buffer or with 5 μmol/L degrasyn for 2 h.

Figure 1. Degrasyn causes the rapid downregulation of c-Myc in MM-1 multiple myeloma cells. A, chemical structure of degrasyn (previously known as WP1130). B, MM-1 cells were treated with cycloheximide (50 μg/mL) for the indicated times, after which cells were harvested, and levels of c-Myc and actin were determined by Western blotting. C, MM-1 cells were incubated with 5 μmol/L degrasyn for the indicated times before cell lysates were prepared and subjected to Western blot analysis for c-Myc and actin levels. D, MM-1 cells were treated with the indicated concentrations of degrasyn for 1 h before lysates were immunoblotted for c-Myc and actin.
Rapid c-Myc down-regulation may involve activation or facilitation of proteolysis. To determine whether proteases were associated with degrasyn-induced regulation of c-Myc, we examined the effect of several protease inhibitors (12, 29) on degrasyn-induced c-Myc degradation in MM-1 cells (Fig. 2A). Pretreatment of MM-1 cells with LLnL, MG-132, or PS341 before incubation with degrasyn strongly reduced c-Myc down-regulation compared with that in cells treated with buffer only. A weaker reduction of c-Myc was observed in cells pretreated with the calpain inhibitor LLnM, which may be due to its previously described weak proteosomal inhibitory activity (30). This effect is minor when compared with that of cells pretreated with more potent and selective proteasome inhibitors (LLnL, MG-132, and PS341). No change in c-Myc down-regulation was noted in cells pretreated with the lysosomal protease inhibitor E-64d, chloroquine, or ammonium chloride. c-Myc depletion by degrasyn was not affected by pretreatment with cycloheximide, suggesting that degrasyn does not require new protein synthesis to induce c-Myc down-regulation. These experiments suggest that specific proteases play a role in the degrasyn-induced depletion of c-Myc (12). Degrasyn also shows c-Myc target selectivity, as the c-Myc complexing protein Max was not affected by degrasyn (Fig. 2B). These results suggest that degrasyn induces the down-regulation of c-Myc protein through activation of a protease or a proteasomal-dependent pathway.

Deletion of amino acids 316 to 378 on c-Myc reduces its destabilization and destruction by degrasyn. c-Myc instability is due primarily to its rapid destruction by ubiquitin-mediated proteolysis (13, 16, 19, 31, 32). The ubiquitin proteolytic pathway involves a specific multistep process that results in proteins being targeted for polyubiquitination and destroyed by the 20S proteasome (9, 33). Many regions of c-Myc have been shown to participate in its stability and proteasomal regulation. The degron situated within the first 147 amino acid residues of the protein has an important signaling role in its ubiquitin-mediated proteasomal degradation. However, regions of c-Myc outside the degron have also been shown to regulate its stability. To determine if degrasyn activates known regulators of c-Myc stability or induces c-Myc degradation by activating a novel signaling pathway, we used a liposomal delivery system to transiently express either wild-type c-Myc (Fig. 3A) or a panel of scanning deletion mutants (ΔΔ-ΔΔ) generated by the deletion of ~60-amino-acid segments from c-Myc (ref. 20; Fig. 3B) in HeLa cells. HeLa cells were used because of their endogenous sensitivity to degrasyn-mediated c-Myc down-regulation and their ability to overexpress c-Myc and its deletion mutants after liposomal transfection. Degrasyn induced the down-regulation of wild-type c-Myc and that of every deletion mutant except the ΔΔF construct (Fig. 3B). This region spans amino acids 316 to 378 of the COOH-terminal region of c-Myc and had not previously been shown to play a major role in c-Myc stability. Deletion of this region increased c-Myc stability in HeLa cells and reduced its sensitivity to destruction by degrasyn-stimulated mechanisms. Expression of another HA-tagged signaling protein (IKKγ) in HeLa cells did not increase its sensitivity to or destruction by degrasyn (Fig. 3C), suggesting that degrasyn targets c-Myc and not the HA epitope.

Mapping of the ΔΔ c-Myc domain for sensitivity to degrasyn. The ΔΔ region of c-Myc (amino acids 316–378) contains a nuclear localization signal (NLS; 320–328) and two of three residues that were recently shown to be phosphorylated by PAK2 (T358 and S373; refs. 23, 24; Fig. 4A). To determine the smallest region of c-Myc necessary to reduce degrasyn-mediated c-Myc destruction, we generated 20-amino-acid deletions within the 60-amino-acid ΔΔ region by site-directed mutagenesis. The HA-tagged wild-type c-Myc, the ΔΔ 60-amino-acid deletion mutant, or three 20-amino-acid deletion mutants (ΔΔ316-335, ΔΔ336-355, or ΔΔ356-378) of c-Myc (Fig. 4B) were expressed after liposomal transfection in HeLa cells. Degrasyn induced the degradation of wild type and ΔΔ336-355 mutant of c-Myc but failed to induce the degradation of the ΔΔ mutant. Deletion of amino acids 316 to 335 and 356 to 378 of c-Myc resulted in reduced sensitivity to degrasyn-mediated c-Myc destruction. These observations suggest that two regions within the ΔΔF region (316–335 and 356–378) form a critical determinant of c-Myc that regulates its stability and its sensitivity to degrasyn in HeLa cells.

The NLS of c-Myc is within amino acids 316 to 335, and two of the three amino acid residues phosphorylated by PAK2 (T358 and S373) are within amino acids 336 to 378. To determine if nuclear localization of c-Myc is necessary for degrasyn activity, we tested the ability of degrasyn to degrade an NLS deletion mutant of c-Myc. Deletion of the NLS failed to inhibit degrasyn-induced degradation of the protein (Fig. 4B). Phosphorylation of the T358, S373, and T400 amino acid residues of c-Myc by PAK2 is known to induce the rapid degradation of c-Myc (24). To test if PAK2-dependent phosphorylation of c-Myc was important for the degrasyn response, we generated T358A/D and S373A/D substitution mutants of c-Myc. Again, degrasyn induced the degradation of these c-Myc substitution mutants (Fig. 4C). These results suggest...
that degrasyn enhances the proteasome-mediated degradation of c-Myc by a novel mechanism targeting c-Myc between amino acid residues 316 to 356 and 356 to 378.

**Degrasyn reduces c-Myc levels in A375 melanoma cells and tumors.** Malignant melanoma has previously been shown to overexpress c-Myc, and re-establishing control of c-Myc levels in these tumors may have therapeutic effects (34–37). Initial screening of a panel of seven melanoma cell lines showed that three cell lines, including A375 cells, were highly sensitive to the antiproliferative/apoptotic effects of degrasyn (A375 IC50 = 1.6 μmol/L). We found the dose-dependent antitumor activity of degrasyn to be aligned with down-modulation of c-Myc protein levels but not other nuclear (p53) or latent (Stat3) transcription factors (Fig. 5A) in A375 cells. Moreover, degrasyn did not inhibit phosphorylated mitogen-activated protein kinase in A375 tumor cells (Fig. 5A), which has previously been shown to be play a role in c-Myc posttranslational modification and stability (38). Because degrasyn-mediated c-Myc down-regulation in A375 cells was similar to that detected in other tumor cells (MM-1 and HeLa), we assessed the in vivo activity of degrasyn against A375 melanoma tumors established in nude mice. For antitumor assessment, Deprnasyn was administered to nude mice at 40 mg/kg every other day for

**Figure 3.** Deletion of amino acids 316-378 of c-myc decreases its sensitivity to destruction by degrasyn. A, the mammalian expression vector for HA epitope-tagged c-Myc was transiently expressed in HeLa cells at the indicated amounts of cDNA. At 24 h after transfection, cells were treated with 5 μmol/L degrasyn for 1 h, subjected to lysis, and analyzed for c-Myc expression by Western blotting with an anti-HA antibody. B, HeLa cells were transfected with the indicated HA-tagged c-Myc deletion constructs (at the indicated amounts of cDNA) for 24 h and then incubated with degrasyn. Cell lysates were prepared and immunoblotted for HA-c-Myc with anti-HA.

C, an expression vector for HA-tagged IKKγ was transfected into HeLa cells, and 24 h later, cells were treated with 5 μmol/L degrasyn for 1 h. Cell lysates were analyzed for IKKγ expression by Western blotting with an anti-HA antibody.

**Figure 4.** Two regions within the ΔF domain of c-Myc are involved in its sensitivity to degrasyn. A, the ΔF domain of c-Myc was subjected to further deletion to determine the smallest region necessary to reduce degrasyn-mediated c-Myc destruction. Constructs included those in which 20 amino acids were deleted within the ΔF of c-Myc, the NLS (amino acids 320–328), and critical serine (S373) or threonine (T358) residues in this region. B and C, HeLa cells were transfected with the indicated deletion constructs of HA-c-Myc (0.75 μg) for 24 h before being treated with degrasyn for 1 h. Cell lysates were prepared and immunoblotted for HA-c-Myc with anti-HA. Actin was immunoblotted and used as the protein loading control.
Degrasyn reduces c-Myc levels and A375 melanoma tumor growth in vitro and in vivo. A, A375 melanoma cells were treated with the indicated concentration of degrasyn for 2 h before analysis of specific protein levels in cell lysates by Western blotting. The IC50 for degrasyn in A375 melanoma cells was 1.6 μmol/L (according to a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay). B, top, A375 tumors were established in nude mice by s.c. injection of 4 × 106 cells. Animals were randomly separated into two groups (five animals per group) and treated with degrasyn or delivery vehicle beginning on day 8 (when tumor volume was ~0.05 cm3). Animals were treated and weighed, and tumor dimensions were measured every other day. No difference in body weight was found between the treated and untreated groups (data not shown). In the first experiment, in which degrasyn treatment began as soon as tumors became detectable (8 days after tumor inoculation), degrasyn significantly suppressed melanoma tumor growth throughout the course of treatment (Fig. 5B, top). Assessment of c-Myc protein levels in tumors extracted from three control-treated mice and three degrasyn-treated mice 2 h after the final injection (day 34) showed significant reductions in c-Myc levels in the degrasyn-treated animals (Fig. 5B, bottom inset). To determine whether tumor size at initiation of therapy would influence the response to degrasyn, tumors were allowed to grow for an additional 9 days, resulting in a 5-fold increase in volume (to ~0.125 cm3) relative to the previous experiments (Fig. 5B, bottom). Degrasyn suppressed tumor growth to an extent similar to that shown in previous experiments involving animals with lower tumor burden. Again, tumor growth inhibition was associated with a reduction in c-Myc protein levels (Fig. 5B, bottom inset). Together, these results show that degrasyn suppresses c-Myc protein levels and suppresses the growth of A375 melanoma tumors in nude mice.

Discussion

In this study, we show that degrasyn induces c-Myc down-regulation is proteasomal-dependently mediated through a novel region not previously associated with its proteasomal degradation. c-Myc protein stability is regulated by a NH2-terminal "degron" that signals c-Myc ubiquitination and a COOH-terminal "stabilon" that stabilizes c-Myc by enabling it to associate with the POZ domain protein Miz or to be sequestered in a subnuclear compartment in which it is stable (12, 17). It was initially assumed that sequences located between the degron and stabilon were dispensable for all aspects of c-Myc function (39, 40). However, the region of c-Myc between amino acid residues 190 and 210 (the D domain) was recently found important for initiating the degrada-
tion of c-Myc by an unknown mechanism (20). Here, phosphorylation of the Thr358, Ser373, and Thr400 amino acid residues by PAK2 has been shown to reduce c-Myc interaction with Max and to induce degradation of c-Myc through an unknown process (23, 24). Here, we characterized a region of c-Myc between amino acid residues 316 to 378 that is important for the small molecule degrasyn to induce proteasomal degradation of c-Myc in tumor cells.

The pattern of phosphorylation of two key amino acid residues Thr358 and Ser373 is critical for ubiquitin-mediated degradation of c-Myc (10) as point mutations at these sites increase the stability of the c-Myc protein (12, 41–43). Here, we show that degrasyn induced 3 weeks without acute toxicity or weight loss (data not shown). Tumors were established by s.c. injection, and degrasyn treatment was initiated at two stages of tumor development. Preliminary dose-finding studies showed that the single-dose degrasyn LD10 was >120 mg/kg (given i.p. or i.v.), whereas multidose toxicity occurred at 80 mg/kg. When administered on alternating days, degrasyn (40 mg/kg) did not cause weight loss or induce acute or cumulative toxicity. Initial pharmacologic assessment of i.v. administered degrasyn (10 mg/kg) revealed a peak plasma level of 0.8 μmol/L and a plasma elimination half-life of 4.5 h. However, daily administration of degrasyn resulted in cumulative toxicity that does not seem to be related to its half-life. Dosing on alternate days suppressed tumor growth in the absence of toxicity or weight loss. More recent studies have shown that degrasyn can be safely administered orally (160 mg/kg every other day) with antitumor activity that is similar to that achieved by 40 mg/kg i.p. dosing (data not shown). In the first experiment, in which degrasyn treatment began as soon as tumors became detectable (8 days after tumor inoculation), degrasyn significantly suppressed melanoma tumor growth throughout the course of treatment (Fig. 5B, top). Assessment of c-Myc protein levels in tumors extracted from three control-treated mice and three degrasyn-treated mice 2 h after the final injection (day 34) showed significant reductions in c-Myc levels in the degrasyn-treated animals (Fig. 5B, top inset). To determine whether tumor size at initiation of therapy would influence the response to degrasyn, tumors were allowed to grow for an additional 9 days, resulting in a 5-fold increase in volume (to ~0.125 cm3) relative to the previous experiments (Fig. 5B, bottom). Degrasyn suppressed tumor growth to an extent similar to that shown in previous experiments involving animals with lower tumor burden. Again, tumor growth inhibition was associated with a reduction in c-Myc protein levels (Fig. 5B, bottom inset). Together, these results show that degrasyn suppresses c-Myc protein levels and suppresses the growth of A375 melanoma tumors in nude mice.
the rapid degradation of c-Myc deletion mutants lacking both Thr36 and Ser36 residues, but mutants devoid of a specific COOH-terminal region (amino acids 316–378) seem to be important for c-Myc stability and necessary for degrasyn-induced c-Myc degradation. The degrasyn-dependent mechanism leading to c-Myc degradation seems to be novel, and we have not detected c-Myc ubiquitination in degrasyn-treated cells (data not shown), suggesting that this compound may induce a form of ubiquitin-independent proteasome-like proteolysis.

The 4F deletion mutant of c-Myc (amino acids 316–378) strongly suppressed the c-Myc down-regulatory activity of degrasyn (Fig. 4B) when compared with smaller deletion mutants from this domain (amino acids 316–335 and 356–378). Interestingly, deletion of amino acids 336 to 355 failed to inhibit the activity of degrasyn, suggesting that a structural motif retained or formed by these regions (316–335 and 356–378) is necessary to engage a complete degrasyn response. The c-Myc NLS (320–328) is situated within the 316–335 region, and two of the PK2 phosphorylation sites (S373 and T389) reside within the 356 to 378 segment. To test whether the degrasyn-induced degradation of c-Myc proceeds through a novel proteasome-dependent pathway mediated through a region of c-Myc that has not been previously defined as a major regulator of c-Myc stability. Recent studies have shown that a region of N-Myc between amino acid residues 317 and 337 may be important for both c-Myc and N-Myc stability. The effect of degrasyn on N-Myc and L-Myc protein levels has not been thoroughly investigated.

Tissue- and organ-specific induction of c-Myc expression induces cellular transformation in a broad variety of cell types, including prostate (46), breast (47), and liver (48), and its induction may underlie the self-renewal ability of hematopoietic stem cells (49). Recent evidence suggests that even transient inhibition of c-Myc can reverse cellular transformation (46–49). Collectively, these findings illustrate the significant potential for compounds that disrupt or inhibit c-Myc to function as antitumor agents or to synergize with conventional chemotherapeutic agents (50). Clearly, small molecular weight antitumor compounds that induce the rapid and specific degradation of c-Myc could have significant therapeutic activity against many human cancers. Because most c-Myc mutations increase protein stability through alterations in the NH2-terminal degron, it may be advantageous to develop agents that target the COOH-terminal region to induce protein turnover. Because of its unique mechanism of action, degrasyn may have the potential to affect c-Myc levels in several human tumors, thereby increasing its therapeutic potential.

To address this potential, we conducted pilot antitumor experiments with A375 melanoma xenografts growing in nude mice. Although degrasyn causes tumor regression and modulates c-Myc levels in vitro and in vivo, it is not clear whether the latter activity is solely responsible for the antitumor activity of this compound. Additional studies are needed to address this possibility. However, the unique mechanism of c-Myc regulation by degrasyn and its antitumor activity support further assessment of its biological and therapeutic properties.

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**References**

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