Mcl-1 Is Required for Melanoma Cell Resistance to Anoikis

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Abstract
Melanoma is a particularly aggressive tumor type that exhibits a high level of resistance to apoptosis. The serine/threonine kinase B-RAF is mutated in 50% to 70% of melanomas and protects melanoma cells from anoikis, a form of apoptosis induced by lack of adhesion or adhesion to an inappropriate matrix. Mutant B-RAF down-regulates two BH3-only proapoptotic proteins, BimEL and Bad. BH3-only proteins act, at least in part, by sequestering prosurvival Bcl-2 family proteins and preventing them from inhibiting the mitochondrial apoptotic pathway. Several Bcl-2 proteins are up-regulated in melanoma; however, the mechanisms of up-regulation and their role in melanoma resistance to anoikis remain unclear. Using RNA interference, we show that depletion of Mcl-1 renders mutant B-RAF melanoma cells sensitive to anoikis. By contrast, minor effects were observed following depletion of either Bcl-2 or Bcl-xL. Mcl-1 expression is enhanced in melanoma cell lines compared with melanocytes and up-regulated by the B-RAF-MEK-extracellular signal-regulated kinase 1/2 pathway through control of Mcl-1 protein turnover. Similar to B-RAF knockdown cells, adhesion to fibronectin protected Mcl-1 knockdown cells from apoptosis. Finally, expression of Bad, which does not sequester Mcl-1, further augmented apoptosis in nonadherent Mcl-1 knockdown cells. Together, these data support the notion that BH3 mimetic compounds that target Mcl-1 may be effective for the treatment of melanoma in combinatorial strategies with agents that disrupt fibronectin-integrin signaling.

Introduction

Anoikis is a form of apoptosis induced by loss of adhesion or adhesion to an inappropriate extracellular matrix (1). The susceptibility of cells to anoikis controls their numbers during development and normal homeostasis. By contrast, malignant cells display resistance to anoikis, a trait that permits their survival at sites distant from the primary tumor. Resistance to various forms of apoptosis is a critical factor contributing to the aggressive nature of melanoma cells. Once this form of skin cancer has metastasized, the clinical prognosis and 5-year survival rates of patients are poor because current treatments are few and often ineffective.

Anoikis is controlled by activation of the mitochondrial apoptotic pathway involving subfamilies of Bcl-2 proteins that differ in their activities (2). Proapoptotic Bcl-2 proteins, Bak and Bax, mediate release of apoptogenic factors from the mitochondrial membrane and activation of the caspase pathway. Bax/Bak activation is modulated by proapoptotic BH3-only proteins including Bad, Bim, NOXA, and PUMA. BH3-only proteins sense cellular damage, but whether they directly activate Bax/Bak or rather act indirectly by sequestering prosurvival Bcl-2 family proteins from inactivating Bax/Bak is currently under debate (3-5). Prosurvival Bcl-2 proteins, such as Bcl-2, Bcl-xL, and Mcl-1, antagonize this pathway through interactions with BH3 domains of BH3-only proteins and Bak/Bax (6). The balance between the expression/activation of the various Bcl-2 family proteins ultimately determines the cellular response.

B-RAF, a serine/threonine kinase, is mutated in 50% to 70% of human melanomas to a form that activates the MEK-extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade (7). We have shown previously that mutant B-RAF and MEK signaling are required for melanoma cell resistance to anoikis (8, 9). Oncogene-mediated resistance to anoikis has also been shown in other tumor cell types, for example, by overexpression of epidermal growth factor receptor in breast cancer cells (10). In melanoma, B-RAF-mediated protection from anoikis is mediated, at least in part, by the down-regulation of two BH3-only proteins, BimEL and Bad (9).

Targeting prosurvival members of the Bcl-2 family holds therapeutic potential for many cancer types. BH3 mimetic compounds that bind to a variety of prosurvival proteins have already been described (11, 12). These small molecules insert into the groove formed by the BH1, BH2, and BH3 domains on the surface of Bcl-2/Bcl-xL and block their inhibitory potential. However, some of these BH3 mimetic compounds target only a subset of Bcl-2 family proteins; thus, it is important to determine which members contribute to resistance to apoptosis in response to different stimuli. Immunohistochemistry studies in melanoma indicate that up-regulation of Bcl-xL and Mcl-1 correlates with melanoma progression (13), but the role of Bcl-2 family proteins in resistance to melanoma anoikis remains unknown. Here, we show that Mcl-1 expression mediates resistance to anoikis in mutant B-RAF human melanoma cells. By contrast, Bcl-2 and Bcl-xL exhibited minor activity in protecting melanoma cells from anoikis. Mcl-1 expression was...
elevated in human melanoma cell lines and its protein stability was regulated by mutant B-RAF/MEK signaling.

Results

Mcl-1 Expression Is Required for Resistance of Melanoma Cells to Anoikis

We have shown previously that mutant B-RAF promotes resistance to anoikis in melanoma cells via down-regulation of BimEL and Bad (8, 9). BH3-only proteins act, at least in part, by sequestering prosurvival Bcl-2 proteins and preventing them from inhibiting the essential proapoptotic proteins, Bak and Bax (14-16). We investigated the role of prosurvival Bcl-2 proteins in resistance to anoikis in mutant B-RAF melanoma cells. We employed a knockdown approach to individually deplete Mcl-1, Bcl-2, and Bcl-XL from WM793 cells that harbor mutant B-RAF (17, 18). Efficient knockdowns were confirmed by Western blotting at both 72 h post-knockdown (Fig. 1A) and 144 h equivalent to the time of cleaved caspase-3 analysis (Supplementary Fig. S1). Bcl-XL knockdowns were less efficient than for Mcl-1 and Bcl-2. Knockdown cells were cultured in serum-free conditions on agar-coated plates for 48 h before assaying for cleaved caspase-3, a marker of activation of the intrinsic apoptosis pathway. In these conditions, Mcl-1 knockdown cells but not control, Bcl-2, or Bcl-XL knockdown cells displayed a large population of cleaved caspase-3-positive cells (Fig. 1B and quantitated in Fig. 1C).

Because cleavage of caspase-3 is an early event leading to apoptosis, we next analyzed Annexin V staining and propidium iodide (PI) uptake as measures of later apoptotic events. Furthermore, to reduce concerns about off-target actions, we used a second, independent small interfering RNA (siRNA) sequence targeting Mcl-1. Both siRNAs reduced Mcl-1 expression without altering the levels of Bcl-2 and Bcl-XL, although Mcl-1 siRNA #11 was more efficient than duplex 12 (Fig. 2A). Importantly, both siRNAs enhanced Annexin V staining and incorporation of PI in suspended WM793 cells (Fig. 2B). Because our serum-free medium conditions may confound

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**FIGURE 1.** Mcl-1 knockdown promotes cleavage of caspase-3 in nonadherent melanoma cells. WM793 cells were transfected with control, Mcl-1, Bcl-2, or Bcl-XL siRNA as indicated. A, Seventy-two hours post-transfection, cell lysates were analyzed by Western blotting for Mcl-1, Bcl-2, or Bcl-XL. Total ERK1/2 was used as a loading control. B, Seventy-two hours post-transfection, WM793 cells were serum starved for 24 h and then replated onto agar-coated dishes in serum-free medium. After 48 h, cells were analyzed for cleaved caspase-3 using flow cytometry. X axis, fluorescence intensity; Y axis, cell counts, with percentages of cells staining positive in each condition. C, Quantitation of the data in B is presented as the average percentage of cells staining positive for cleaved caspase-3 from three independent experiments.
the analysis, we performed similar experiments in medium containing 2% serum and insulin. Mcl-1 knockdown with either siRNA was susceptible to anoikis in serum-containing and growth factor-containing medium albeit at a reduced extent compared with serum-free conditions (Supplementary Fig. S2). To extend these findings to more than one melanoma line, we performed similar experiments in A375 cells, which also harbor mutant B-RAF. Selective knockdown of Mcl-1 led to increased Annexin V staining and PI incorporation in suspended A375 cells (Fig. 2C and D). We also performed Mcl-1 knockdowns in Sbcl2 cells that are wild-type for B-RAF but harbor a N-RAS mutation (18,19). Sbcl2 cells display high sensitivity to anoikis that was further enhanced following Mcl-1 knockdown (Supplementary Fig. S3). These data show that depletion of Mcl-1 renders melanoma cells susceptible to anoikis.

Regulation of Mcl-1 in Melanoma Cells

Increased staining for Mcl-1 has been observed in primary and metastatic melanomas (13). We analyzed Mcl-1 levels in a panel of melanoma cells compared with human melanocytes. All the melanoma lines harbor mutant B-RAF with the exception of Sbcl2, which is mutant for N-RAS (18,19). Mcl-1 protein levels were enhanced in the majority of these cells compared with melanocytes (Fig. 3A). The MEK-ERK1/2 pathway is elevated in melanoma cell lines (Fig. 3A; ref. 17, 18) and Mcl-1 levels decreased following inhibition of MEK with U0126 (Fig. 3B; Supplementary Fig. S3C). No alterations in the levels of Bcl-2 and Bcl-XL were detected in these conditions. Additionally, we generated WM793 cell lines expressing inducible control or B-RAF short hairpin RNAs (shRNA). Treatment of B-RAF shRNA cells with doxycycline led to an efficient decrease in the levels of B-RAF and concomitant decrease in phospho-ERK1/2 (Fig. 3C). No effect was observed in control shRNA WM793 cells. In support of MEK inhibitor experiments, inducible knockdown of B-RAF in WM793 cells led to a decrease in Mcl-1 expression (Fig. 3D).

Mcl-1 levels can be regulated by the proteasome (20, 21). Treatment of WM793 cells with proteasomal inhibitors increased expression of Mcl-1 (Fig. 4A). To determine whether turnover of Mcl-1 was regulated by MEK-ERK1/2, we next analyzed protein turnover in cycloheximide chase experiments in the absence or presence of MEK inhibitor. Mcl-1 levels were efficiently decreased in U0126-treated WM793 cells compared with DMSO controls over a 6-h time course (Fig. 4B). Similarly, turnover of Mcl-1 was increased in U0126-treated A375 cells (Fig. 4C). These data show that depletion of Mcl-1 renders melanoma cells susceptible to anoikis.

**FIGURE 2.** Mcl-1 knockdown sensitizes WM793 and A375 cells to anoikis. A, WM793 cells were transfected with control, Mcl-1^{#11}, or Mcl-1^{#12} siRNA. Cell lysates were analyzed by Western blotting for Mcl-1, Bcl-2, Bcl-XL, and ERK1/2. B, Following transfection and serum starvation, WM793 cells were replated onto agar-coated dishes for 48 h in serum-free medium. Harvested cells were analyzed for Annexin V staining and PI uptake. The main trace shows Annexin V-FITC staining versus relative cell number. Inset, Annexin V-FITC staining versus PI incorporation. C and D, As in A and B, except that A375 cells were used. Representative traces from one of three independent experiments.
cells compared with control treatment, although basal turnover of Mcl-1 was more rapid in these cells compared with WM793 (Fig. 4C). Mcl-1 levels were up-regulated in U0126-treated cells following proteasomal inhibition (Fig. 4D and E). Phosphorylation of Mcl-1 within proline-glutamate-serine-threonine sequences regulates Mcl-1 protein turnover (22-24). Levels of phospho-Mcl-1 were also up-regulated following MG132 treatment alone. Consistent decreases in the levels of phospho-Mcl-1 were observed following MG132/U0126 treatment compared with MG132 alone (22-24). The incomplete inhibition of phosphorylation was expected because multiple phosphorylation sites are recognized by this antibody. Together, these data show that Mcl-1 protein turnover is regulated by the MEK-ERK1/2 pathway in mutant B-RAF melanoma cells likely through phosphorylation within the proline-glutamate-serine-threonine domain.

Survival of Mcl-1 Knockdown Cells Is Adhesion-Dependent

We have shown previously that adhesion to fibronectin protects mutant B-RAF-depleted melanoma cells from apoptosis (8). Next, we determined whether fibronectin protected Mcl-1 knockdown cells following proteasomal inhibition (Fig. 4D and E). Phosphorylation of Mcl-1 within proline-glutamate-serine-threonine sequences regulates Mcl-1 protein turnover (22-24). Levels of phospho-Mcl-1 were also up-regulated following MG132 treatment alone. Consistent decreases in the levels of phospho-Mcl-1 were observed following MG132/U0126 treatment compared with MG132 alone (22-24). The incomplete inhibition of phosphorylation was expected because multiple phosphorylation sites are recognized by this antibody. Together, these data show that Mcl-1 protein turnover is regulated by the MEK-ERK1/2 pathway in mutant B-RAF melanoma cells likely through phosphorylation within the proline-glutamate-serine-threonine domain.

Expression of Bad Enhances Susceptibility of Mcl-1 Knockdown Cells to Anoikis

B-RAF knockdown in combination with Mcl-1 knockdown slightly enhanced susceptibility to anoikis (Fig. 5). We have shown previously that the BH3-only protein Bad is up-regulated following B-RAF knockdown and is required, in part, for sensitivity to anoikis in B-RAF knockdowns (8, 9). Work from others has shown that Bad does not target Mcl-1 (14-16); thus, we tested whether Bad expression enhanced apoptosis in Mcl-1 knockdown cells. We generated an inducible system to express wild-type Bad following addition of doxycycline to cells (Fig. 6A). Expression of Bad alone increased slightly the sensitivity of WM793 to anoikis, consistent with our previous study (8, 9). Additionally, Bad expression further enhanced the susceptibility of Mcl-1 knockdown cells to anoikis (Fig. 6B). These data suggest

![FIGURE 3.](image-url)

**FIGURE 3.** Mcl-1 is highly expressed in melanoma cells. A, Protein cell lysates from human melanocytes (NHEM) and melanoma cells (Sbc2, WM5, WM783, WM278, WM115, WM1205, WM9, A375, SK-MEL-28, and SK-MEL-5) were analyzed for Mcl-1, phospho-ERK1/2, and total ERK1/2 levels. B, WM793 cells were treated with U0126 for 5 h and lysates were analyzed by Western blotting. C, WM793TR-Ctl shRNA and WM793TR-B-RAF shRNA cell lines were untreated or treated with 100 ng/mL doxycycline for 72 and 96 h. Cell lysates were analyzed by Western blotting for B-RAF, phospho-ERK1/2, and total ERK1/2. D, Control and Mcl-1 knockdown WM793 cells and WM793TR-Ctl shRNA and WM793TR-B-RAF shRNA treated with doxycycline for 96 h were analyzed by Western blotting for levels of Mcl-1, B-RAF, and actin. Levels of Mcl-1 normalized to actin are indicated for each sample.
that mechanisms in addition to inactivation of Mcl-1 contribute to melanoma cell susceptibility to anoikis.

**Discussion**

Melanoma is renowned for its resistance to apoptosis. In this study, we examined the role of prosurvival Bcl-2 family members. These studies are the first to show a role for Mcl-1 in melanoma cell resistance to anoikis. Our data add to growing evidence implicating Mcl-1 in melanoma. Antisense oligonucleotide/siRNA strategies to down-regulate Mcl-1 increases melanoma cell sensitivity to apoptosis induced by dacarbazine treatment *in vivo* (25), exposure to ionizing radiation *in vitro* (26), the proteasome inhibitor bortezomib (27), and endoplasmic reticulum stress (28). Additionally, a small-molecule BH3 mimic, obatoclax, which targets Mcl-1, renders melanoma cells sensitive to the Bcl-2/Bcl-XL/Bcl-WL selective antagonist, ABT-737, and to bortezomib (29, 30). Thus, Mcl-1 may underlie resistance to several forms of proapoptotic signals.

Our studies focus on anoikis, a form of apoptosis induced by loss of or inappropriate adhesion. We have shown previously that B-RAF knockdown was associated with the up-regulation of two proapoptotic proteins, BimEL and Bad (9). BimEL is known to bind Mcl-1 (5, 11, 31); thus, the effects of up-regulated BimEL following B-RAF knockdown are likely to prevent Mcl-1 actions on Bax/Bak. Bad, however, does not bind Mcl-1 and likely further enhances sensitivity to anoikis in Mcl-1 knockdown cells. This result indicates the multifactorial mechanism underlying B-RAF-dependent resistance to anoikis. Similar to our findings with B-RAF depletion, adhesion to fibronectin was protective for Mcl-1 knockdown cells. The integrins αvβ3 and α4β1 are up-regulated in invasive melanomas (32) and both are capable of binding fibronectin in addition to

![FIGURE 4](https://www.mcr.aacrjournals.org/figure4.png)

**FIGURE 4.** Mcl-1 protein turnover is regulated by B-RAF-MEK signaling. **A**, WM793 cells were treated with 10 μmol/L MG132 or 50 μmol/L ALLN for 5 h. Cell lysates were analyzed by Western blotting. **B**, WM793 cells were treated with 10 μg/mL cycloheximide for 1 h after which one culture dish was taken as time 0. Cells were then treated with either DMSO or 5 μmol/L U0126 for the indicated time. Cell lysates were analyzed by Western blotting for Mcl-1 and actin (loading control). **C**, As in **B**, except A375 cells were used. **D**, WM793 cells were treated for 6 h with 10 μmol/L MG132 and/or 5 μmol/L U0126. Cell lysates were analyzed by Western blotting for levels of total and phospho-Mcl-1 and total and phospho-ERK1/2. **E**, As in **D**, except that A375 cells were used. Representative blots from one of three independent experiments.
other ligands (33). Fibronectin deposits are found in the dermis of human skin (34) and also at premetastatic niche sites (35). Thus, combinatorial targeting of fibronectin integrins and Mcl-1 (or B-RAF) may represent an effective strategy to target melanoma cells.

Additionally, our study shows that Mcl-1 expression is up-regulated in multiple human melanoma lines compared with normal human epidermal melanocytes. However, there is not an exact correlation between Mcl-1 levels and resistance to anoikis, indicating that mechanisms in addition to up-regulation of Mcl-1 exist. Nevertheless, these data support immunohistochemistry studies showing that Mcl-1 expression is increased in primary and metastatic melanomas compared with benign melanocytic lesions (13). Mcl-1 is regulated by the proteasome through the action of E3 ubiquitin ligases including Mule and β-TRCP1/2 (20, 21, 36). It is possible that high expression of Mule and/or β-TRCP1/2 levels in cell lines such as WM9 and SK-MEL-28 mediates the low Mcl-1 levels. GSK3β-mediated Ser159 phosphorylation of Mcl-1 leads to increased ubiquitylation and proteasomal degradation (24). By contrast, phosphorylation of Thr163 in response to ERK1/2 activation inhibits the proteasomal turnover of Mcl-1 (23). Thus, it is likely that decreased phosphorylation of Mcl-1 following B-RAF knockdown promotes Mcl-1 turnover. Although our findings show proteasomal regulation of Mcl-1 levels, we cannot rule out that B-RAF regulates Mcl-1 expression in part through control of mRNA levels.

In summary, our findings show that Mcl-1 is required for protection from anoikis in melanoma and Mcl-1 protein turnover is regulated by the B-RAF-MEK pathway. These studies underscore the importance of targeting Mcl-1, likely in combination with integrin antagonists, as a possible therapeutic strategy for melanoma.

Materials and Methods

Cell Culture

All melanoma cells, except A375, were routinely subcultured in MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% fetal bovine serum, and 5 μg/mL insulin. A375 cells were cultured in DMEM containing 10% fetal bovine serum. Isolation of neonatal human melanocytes has been described elsewhere (17).

siRNA Knockdowns

WM793 cells were transfected with siRNA at a final concentration of 25 nmol/L using Oligofectamine (Invitrogen) as described previously. A375 cells were electroporated using program K-017 on a Nucleofector (Amaxa Biosystems). The siRNA sequences (Dharmacon) used were control: UAGCGACUAAACACAUAUU, Mcl-111: GCAUCGAACCAUUAGCAGAUU, Mcl-112: GCUGAACUUGAGACAUUU, Bel-2: GGGAGAUGAUGAGAAGAUAUU, and Bel-XL: GGAGAUGCAGGUAUUGGUGUU.

Western Blotting

Western blotting was completed as described previously (8, 9). The following primary antibodies were purchased from Santa Cruz Biotechnology: Mcl-1, B-RAF, and ERK1. Bad, Bcl-XL, phospho-ERK (Thr202/Tyr204), and phospho-Mcl-1 (Ser159/Thr163) were obtained from Cell Signaling Technology. Bcl-2 antibody was purchased from BD Biosciences. Anti-actin was purchased from Sigma-Aldrich.

**FIGURE 5.** Adhesion to fibronectin protects Mcl-1 knockdown cells from apoptosis. A. Doxycycline-treated WM793TR-Ctl shRNA and WM793TR-B-RAF shRNA cells were transfected with either control or Mcl-1 siRNAs. Following transfection and serum starvation, WM793 cells were replated onto agar or fibronectin-coated dishes for 48 h in serum-free medium. Harvested cells were analyzed for cleaved caspase-3 staining by flow cytometry. B. Quantitation of cleaved caspase-3 staining from three independent experiments.
A. WM793TR-Bad cells were treated for 72 h with 100 ng/mL doxycycline. Lysates were analyzed by Western blotting for Bad expression and ERK1/2 as a loading control.

B. WM793TR-Bad cells were transfected with control or Mcl-1 siRNA and used in anoikis assays. Harvested cells were analyzed for Annexin V staining and PI uptake. Representative results from one of three independent experiments.

Apoptosis Assays

Cells were serum starved for 24 h before replating onto either 10 μg/mL fibronectin or 1% bactoagar for 48 h in serum-free MCDB 153 medium containing 0.5% bovine serum albumin. Cells were processed for either cleaved caspase-3 flow cytometry, as we have described previously (8, 9), or Annexin V/PI staining. For the latter, cells were harvested off agar, washed in PBS/0.1% bovine serum albumin, and resuspended in 100 μL binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2] at a concentration of 1 x 10⁶ cells/mL. Cells were then incubated for 15 min at ambient temperature with Annexin V-FITC (BD Biodyes) and PI (final concentration of 100 μg/mL; Molecular Probes). After incubation, cells were diluted 1:4 in binding buffer and analyzed by flow cytometry.

Recombinant Lentiviral Transduction

Inducible shRNA knockdown of B-RAF was achieved using the BLOCK-iT lentiviral expression system (Invitrogen) according to the manufacturer’s instructions. The generation of WM793TR cells has been described (9). The sequences for the B-RAF shRNA oligos were 5’-CACCAGAGAGCCT-CAAGAGTAAATCCAAGAGTTACTCTTGAGGTTCCTG-3’ and 5’-AAACACAGACCTCAAGAGTTACTCTTGAGGTTCCTG (italicized is the hairpin). Oligonucleotide sequences for the control shRNA were 5’-CACCGTACGACTAAACACATCAATTCAAGAGTTGATTTGTTAGTCCTA and 5’-AAATAGCCACTAAACACATCAATTCAAGAGTTGATTTGTTAGTCCTA. Oligos were annealed and ligated into the pENTR/H1/TO vector and analyzed for errors by DNA sequencing. Correct constructs were then recombined into the pLenti4/BLOCK-iT-DEST vector. Bad cDNA in pENTR/3C vector (gift from Dr. Matthew VanBrocklin, Van Andel Research Institute) was recombined with pLenti4/TO/V5-DEST using the LR Clonase II kit. Stop codons from cDNAs were maintained to omit the inclusion of a V5 epitope tag contained within the vectors. Lentiviral particles were generated, as described previously, and used to transduce WM793TR cells. Transduced cells were selected with zeocin over 2 weeks. Inducible expression of the transgene was obtained by treatment of cultures with doxycycline at a final concentration of 100 ng/mL.

Protein Turnover Assays

For cycloheximide chase assays, cells were pretreated with 10 μg/mL cycloheximide (Sigma) for 1 h. Zero time point samples were lysed. U0126 (5 μmol/L) or DMSO (vehicle control) was added and cells incubated for 1, 2, 4, and 6 h. In other experiments, the proteasome inhibitors MG132 (Calbiochem) and ALLN were used at final concentrations of 10 and 50 μM, respectively.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Meenhard Herlyn (The Wistar Institute) for WM793 melanoma cells, Dr. Matthew VanBrocklin (Van Andel Research Institute) for Bad vector, and the flow cytometry facility at Albany Medical College for use of the FACScan and FlowJo software.

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