Differentiation-Related Gene-1 Decreases Bim Stability by Proteasome-Mediated Degradation

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Abstract

Drg1 was identified as a differentiation-related, putative metastatic suppressor gene in human colon and prostate cancer. Its expression is associated with resistance to irinotecan (CPT-11) therapy in preclinical colorectal cancer models both in vitro and in vivo. However, the functional significance of Drg1 in these processes is unknown. We have shown for the first time that Drg1 directly binds to the BH3-only proapoptotic protein Bim. Depletion of Drg1 by small interfering RNA induced up-regulation of Bim and its accumulation in the mitochondria, which correlated with loss of mitochondrial membrane potential and induction of apoptosis in cells exposed to SN-38. Further analyses revealed that Drg1 promotes degradation of Bim through the Cullin2/ElonginB-CIS ubiquitin-protein ligase complex. Conversely, in the absence of Drg1, Bim was stabilized and bound more abundantly to Hsp70. These results show that Drg1 renders cancer cells more resistant to chemotherapy through enhanced proteasome-mediated Bim degradation.

Introduction

Drg1/Cap43/NDRG1 is a member of the NDRG family of four genes that share 57% to 65% amino acid identity. Although these proteins have been proposed to possess the α/β hydrolase fold, they do not have hydrolase activity (1, 2). Drg1 contains three unique tandem repeats of 10 hydrophilic amino acids near the COOH terminus. It was previously identified through differential screening techniques during stress response, hormone responses, COOH terminus. It was previously identified through differential screening techniques during stress response, hormone responses, and its high expression is an indicator of poor prognosis in hepatocellular carcinoma (16). Also, Drg1 was observed to increase during colorectal carcinogenesis, suggesting that Drg1 might play a role in tumor progression (17).

We have previously reported that in Hct116 human colon cancer cells, Drg1 is transcriptionally induced by SN-38, the active metabolite of the topoisomerase I poison irinotecan (CPT-11; ref. 18). The suppression of Drg1 in Hct116 cells using specific antisense constructs rendered these cells sensitive to SN-38 in vitro and to CPT-11 in vivo with enhanced apoptosis. In contrast, overexpression of Drg1 in SW620 colon cancer cells, which have low endogenous expression of Drg1, resulted in resistance to SN-38 in vitro and to CPT-11 in vivo. In a clinical analysis of 131 patients with metastatic colon cancer, 40% of the tumor cells in the liver lesions stained positively for Drg1. In a subset analysis of patients who went on to receive CPT-11 therapy, patients with low Drg1 expression (<30% cells staining) in their liver metastases remained on irinotecan-based therapy for 9.3 months, whereas those with high Drg1 expression (>30%) remained on therapy for 6.8 months, suggesting an increased sensitivity to CPT-11 in the Drg1 low patient population (19). These results indicate that Drg1 is highly expressed in both primary and metastatic colon cancers. Furthermore, Drg1 may play a functional role in the sensitivity to CPT-11, such that increased Drg1 expression results in a decrease in CPT-11 sensitivity. However, the mechanism by which Drg1 renders these cells resistant to CPT-11 remains unknown. Here, we report that Drg1 interacts with the proapoptotic BH3-only protein Bim (20) and negatively regulates Bim protein stability by mediating its binding to the proteasome. Therefore, down-regulation of Drg1 stabilizes Bim and results in increased sensitivity to CPT-11.

Materials and Methods

Cell culture. Human Hct116 colon carcinoma cells were purchased from the American Type Culture Collection; cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin; and maintained at 37°C in 5% CO2. The human gastric cancer cell line MKN74 was supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). SK-Mel-173 and SK-Mel-19 melanomas were obtained from Dr. A. Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY), and OCM3 ocular melanoma was from Robert Folberg (University of Illinois, Chicago, IL). Cells were treated with SN-38 (5 mmol/L stock; supplied by Pfizer, Inc.). For protein stability experiments, cells were treated with 10 μmol/L cycloheximide (Sigma) for up to 5 h.

RNAi-mediated gene knockdown. Small interfering RNA (siRNA) against Drg1 and Bim (21) were purchased from Dhharmacon, Inc. The
Drg1 siRNA sequences were GGAGUCCUUCAACAGUUUG (22) and GCAUUAAUGGCAUGGGAAC (23). Control siRNA, which consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA, and Hsp70 siRNA were from Santa Cruz Biotechnology. They were transfected in Hct116 cells using Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer’s instructions. The HA-tagged Drg1 siRNA recognition site silent mutant (called HA-Drg1-M) was generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) with the oligonucleotide GCTTCCTGGAGTACTCCAGCATTTGGCGTC. The plasmid was authenticated by DNA sequencing and transfected in cells 24 h after the siRNAs with FuGENE 6 (23). The empty HA-CMV vector (Clontech) was used as control.

**Apoptosis assays.** Apoptosis was measured using Annexin V-FITC Apoptosis detection Kit II (BD Pharmingen) following manufacturer’s instructions. Both adherent and floating cells were collected and resuspended in Annexin V binding buffer. The Annexin V–positive population was analyzed on a FACScan (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star, Inc.). One representative experiment is shown. Apoptosis was also measured by quantitative fluorescence microscopy (QFM) with 4,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.) for nuclear chromatin staining. Mitochondrial membrane potential was monitored using MitoProbe DiIC1(5) Assay Kit (Molecular Probes, Invitrogen Detection Technologies) and analyzed by flow cytometry. The cyanine dye DiIC1(5) at 100 nmol/L penetrates the cytosol of eukaryotic cells and accumulates primarily in mitochondria with active membrane potentials. Flow cytometry analysis reveals a decrease in fluorescence in cells with disrupted mitochondrial membrane potential.

**Immunoblotting and immunoprecipitation.** Cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics) and 1 mmol/L NaVO₃. Total protein concentration of the lysates was measured by the Bio-Rad protein assay (Bio-Rad Laboratories), and equal amounts of protein were loaded on 4% to 12% PAGE gels (Invitrogen). The membranes were probed with Drg1 (graciously supplied by Therese Commes, University Montpellier II, Montpellier Cedex, France), Bim (Calbiochem), Bak (Pharmingen), Bid (Cell Signaling), Bax, ubiquitin, and elongin B (Santa Cruz Biotechnology), Noxa (Imgenex), Hsp70 (Stressgen), Cullin 2 (Abcam), and α-tubulin (Upstate Biotechnology) antibodies. For immunoprecipitation experiments, equivalent amount of proteins from the cleared lysates were incubated with antibodies for 3 h at 4°C. Then, 30 μL of agarose beads (Upstate Biotechnology) were added for an additional hour. Immunocomplexes were washed in lysis buffer and suspended in 4× SDS sample buffer and Western analysis of bound proteins. The mitochondrial and cytoplasmic fractions were prepared using the Mitochondria Isolation Kit (Pierce Biotechnology).

**Glutathione S-transferase binding assays.** The map of the various Drg1 deletion constructs used in these studies is shown in Fig. 5B. For these experiments, the full-length Drg1 cDNA was amplified by PCR (Supplementary Methods). The products were digested with EcoRI and XhoI, and directionally cloned in pGEX-6P-2 vector (Pharmacia). The expression of the glutathione S-transferase (GST)-Drg1 fragments was confirmed by SDS-gel electrophoresis and Coomassie blue staining. In binding assays, purified GST fusion proteins bound to glutathione beads were incubated with recombinant Bim protein (R&D Systems) for 2 h at 4°C in 250 μL of binding buffer containing 20 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.05% Tween 20, and 1 mmol/L DTT. GST beads were used as control. The beads were washed thrice with binding buffer and collected by centrifugation. The bound Bim protein was eluted in SDS sample buffer, subjected to SDS-PAGE, and detected by immunoblotting.

Results

**Down-regulation of Drg1 induces Bim and sensitizes Hct116 cells to SN-38.** It has been previously shown that inhibition of...
endogenous Drg1 expression in Hct116 cells by stable expression of an antisense Drg1 cDNA increased the sensitivity of cells to undergo apoptosis by the active metabolite of CPT-11, SN-38 (18). A siRNA-based approach was used to selectively inhibit Drg1 expression in Hct116 cells before exposure to 250 nmol/L SN-38 for 24 h and examined for apoptosis by Annexin V/propidium iodide staining. Under these conditions, apoptosis increased from 19.1% with control siRNA to 35.5% (Fig. 1A) following selective suppression of Drg1 (Fig. 1D). This was further evaluated by DAPI staining in QFM assays. Apoptosis was induced by SN-38 in a time- and dose-dependent manner in Hct116 cells transfected with Drg1 siRNA compared with control siRNA (Fig. 1B and C). Whereas Drg1 expression was decreased with two nonoverlapping siRNAs, we also found a substantial increase in the expression of the proapoptotic protein Bim, especially the BimEL form (Fig. 1D). This effect on Bim was reversed by reintroducing Drg1 in cells transfected with siRNA using a HA-tagged Drg1 construct (HA-Drg1-M) carrying third-base silent mutations within the siRNA recognition site, thus excluding off-target interactions of the siRNA (Fig. 1E). The expression of other proapoptotic proteins, including Bax, Bid, Bak, and Noxa, did not change after Drg1 siRNA transfection (Fig. 2A), suggesting a relative degree of specificity for Bim induction. Also, Drg1 siRNA did not affect the expression of antiapoptotic proteins Bcl-2 and Mcl-1, and SN-38 treatment did not change the expression of either (Fig. 2B). Bim expression was also analyzed in a panel of different cancer cell lines. Down-regulation of Drg1 induced Bim only in Drg1-expressing cells (MKN-74 and Mel-173), whereas no induction of Bim was observed in cells with undetectable Drg1 (Mel-19 and OCM3; Fig. 2C), further confirming the specificity of the Drg1 siRNA. To determine whether Bim expression mediated the increase in apoptosis by SN-38 following Drg1 down-regulation, a specific Bim siRNA was transfected in Hct116, alone or together with Drg1 siRNA (Fig. 2D, right). Bim knockdown inhibited the induction of apoptosis by 50% in cells cotransfected with Drg1 siRNA, whereas it did not significantly

Figure 2. Drg1 silencing induces Bim. A, Hct116-transfected cells were analyzed for expression of Drg1, Bim, Bax, Bid, Bak, and Noxa. B, Western blot analysis of siRNA-transfected Hct116 cells treated with 250 nmol/L SN-38 for Bcl-2 and Mcl-1 expression. C, four cell lines were transfected with control and Drg1 siRNA and analyzed for Drg1 and Bim expression. Tubulin is shown to confirm equal protein loading. Each blot is representative of multiple independent experiments. D, Hct116 cells were transfected with the Drg1 and Bim siRNAs alone or combined, and analyzed by immunoblotting (inset). Apoptosis was measured by QFM after 24 h of SN-38 treatment. Columns, mean of three independent experiments performed in duplicates. *, P < 0.003 versus Drg1 siRNA alone.

endogenous Drg1 expression in Hct116 cells by stable expression of an antisense Drg1 cDNA increased the sensitivity of cells to undergo apoptosis by the active metabolite of CPT-11, SN-38 (18). A siRNA-based approach was used to selectively inhibit Drg1 expression in Hct116 cells before exposure to 250 nmol/L SN-38 for 24 h and examined for apoptosis by Annexin V/propidium iodide staining. Under these conditions, apoptosis increased from 19.1% with control siRNA to 35.5% (Fig. 1A) following selective suppression of Drg1 (Fig. 1D). This was further evaluated by DAPI staining in QFM assays. Apoptosis was induced by SN-38 in a time- and dose-dependent manner in Hct116 cells transfected with Drg1 siRNA compared with control siRNA (Fig. 1B and C). Whereas Drg1 expression was decreased with two nonoverlapping siRNAs, we also found a substantial increase in the expression of the proapoptotic protein Bim, especially the BimEL form (Fig. 1D). This effect on Bim was reversed by reintroducing Drg1 in cells transfected with siRNA using a HA-tagged Drg1 construct (HA-Drg1-M) carrying third-base silent mutations within the siRNA recognition site, thus excluding off-target interactions of the siRNA (Fig. 1E). The expression of other proapoptotic proteins, including Bax, Bid, Bak, and Noxa, did not change after Drg1 siRNA transfection (Fig. 2A), suggesting a relative degree of specificity for Bim induction. Also, Drg1 siRNA did not affect the expression of antiapoptotic proteins Bcl-2 and Mcl-1, and SN-38 treatment did not change the expression of either (Fig. 2B). Bim expression was also analyzed in a panel of different cancer cell lines. Down-regulation of Drg1 induced Bim only in Drg1-expressing cells (MKN-74 and Mel-173), whereas no induction of Bim was observed in cells with undetectable Drg1 (Mel-19 and OCM3; Fig. 2C), further confirming the specificity of the Drg1 siRNA. To determine whether Bim expression mediated the increase in apoptosis by SN-38 following Drg1 down-regulation, a specific Bim siRNA was transfected in Hct116, alone or together with Drg1 siRNA (Fig. 2D, right). Bim knockdown inhibited the induction of apoptosis by 50% in cells cotransfected with Drg1 siRNA, whereas it did not significantly
change the apoptosis induced by SN-38 when Bim siRNA was transfected alone (Fig. 2D, left). These results suggest that Bim induction plays a direct role in the sensitivity to SN-38 only when Drg1 is down-regulated.

BH3-only proteins have been reported to localize in different cellular compartments, including the mitochondria, cytosol, and microtubules, to sense different sources of stress (24). However, mitochondrial targeting of Bim is required for induction of apoptosis (25). Thus, we analyzed the subcellular localization of BiminHct116cellswithorwithoutDrg1.AsshowninFig.3A,with Drg1 suppression, there was a marked increase of Bim in the mitochondria. OxPhos is shown to confirm equal loading and purity of the mitochondrial fractions. This induction of Bim correlated with a 2-fold increase in mitochondrial membrane depolarization when Drg1-siRNA cells were exposed to SN-38 treatment, compared with control siRNA cells (Fig. 3B).

**Drg1 promotes Bim degradation through the proteasome.**

To determine whether Drg1 affects Bim expression at the transcriptional level, we performed real-time PCR in Hct116 cells 48 h after siRNA transfections. This analysis revealed an average increase in Bim mRNA levels of 1.2-fold (data not shown), which could not account for the 5-fold increase of Bim observed at the protein level. Furthermore, we also examined the RNA stability of Bim by treating siRNA-transfected cells with actinomycin D, and we found no difference in the kinetics of Bim RNA degradation (data not shown). Hence, we concluded that Drg1 depletion does not affect RNA transcription or stability of Bim.

Next, we tested whether Drg1 loss affected Bim protein stability. Hct116 cells were transfected with the siRNAs for 48 hours, treated with cycloheximide, and analyzed for Bim protein expression over the time indicated. Whereas in Drg1-expressing control cells Bim was degraded after 3 hours of treatment, in Drg1-depleted cells Bim levels were more stable (Fig. 4A; Supplementary Figs. S1B and S1C), suggesting that the presence of Drg1 favors Bim protein degradation. It has been previously reported that Bim is regulated by posttranslational mechanisms, such as extracellular signal-regulated kinase–mediated phosphorylation, leading to increased Bim ubiquitination and proteosomal degradation (26). We found no change in the phosphorylation of Bim after Drg1 down-regulation (data not shown). Therefore, we investigated whether Drg1 could affect Bim ubiquitination. Cell lysates from siRNA-transfected Hct116 cells were immunoprecipitated with a Bim antibody, followed by immunoblotting using ubiquitin or Bim antibodies. Bim was ubiquitinated in control siRNA–transfected cells. However, the ubiquitinated forms decreased in Drg1-depleted cells, especially 72 hours after transfection, whereas immunoprecipitated Bim was elevated (Fig. 4B, bottom). Hence, the lack of Drg1 directly accounts for the decrease in Bim ubiquitination and its rate of protein degradation.

To identify Bim-interacting partners involved in its degradation, we performed a series of immunoprecipitation experiments. First,
we found that Bim coimmunoprecipitated with Drg1 but not with a control IgG antibody (Fig. 4C, top). Previous findings showed that Drg1 interacted with the heat shock cognate protein 70 (Hsc70; ref. 27). Thus, we tested whether Bim could also bind to the heat shock family members Hsc70/Hsp70 and whether Drg1 could affect Bim stability through these interactions. We found a remarkable increase in the binding of Bim to Hsp70 in cells transfected with Drg1 siRNA (Fig. 4C, bottom right). Also, the inverse immunoprecipitation with a Bim antibody revealed binding to Hsp70 (Supplementary Fig. 5A). We could also show that down-regulation of Hsp70 by siRNA induced a decrease in Bim stability when cells were treated with cycloheximide, especially in Drg1 siRNA–cotransfected cells (Supplementary Figs. S1B and S1C).

However, although Hsp70 seemed to protect Bim in the absence of Drg1, it does not explain how Bim is degraded in the presence of Drg1. More recently, it has been reported that RACK1 promotes Drg1, it does not explain how Bim is degraded in the presence of Drg1.

Bim directly binds to Drg1 at the carboxyl terminus. To further characterize the Drg1-Bim interaction, Drg1 was expressed as a GST fusion protein and used in binding assays with recombinant Bim. As shown in Fig. 5A, recombinant Bim bound to immobilized GST-Drg1 in a dose-dependent manner but not to GST alone. Next, a series of GST-tagged Drg1 deletion mutants were generated (Fig. 5B) to identify the region of the Drg1 sequence responsible for the interaction with Bim. In agreement with the immunoprecipitation experiments, Bim bound to full-length Drg1 and also to a fragment lacking the amino terminus (∆N-Drg1), whereas it did not bind to ∆1-Drg1, ∆2-Drg1, ∆3-Drg1, or GST (Fig. 5C). These results suggest that Bim directly interacts with Drg1 at amino acids 338 to 394.

Discussion
We have previously reported that suppression of Drg1 increases sensitivity to CPT-11 in Hct116 colon cancer–bearing xenografts and results in increased sensitization to SN-38–induced apoptosis in this same cell line (18). Its relevance to CPT-11 resistance in colon cancer therapy has also been suggested by several recent studies (19, 29). The pan-CDK inhibitor flavopiridol has been shown to suppress the transcriptional induction of Drg1 by SN-38 and enhance SN-38–induced apoptosis in Hct116 colon cancer cells in vitro and increase the efficacy of CPT-11 in vivo (18). The importance of Drg1 down-regulation by flavopiridol to the sensitivity to CPT-11 has been evaluated in a phase I clinical trial (30). The results from serial biopsies indicated that clinical benefit to therapy was only observed in patients who exhibited a decrease or no induction in Drg1 protein expression. These findings are consistent with emerging data suggesting that Drg1 plays an essential role in the resistance to CPT-11 (31), and suppressing its expression both by pharmacologic or molecular means could increase sensitization to this agent both in vitro and in vivo across a
spectrum of tumor types. Nevertheless, the function of this protein relative to chemotherapy resistance has remained essentially unknown.

Here, we provide the first evidence of the mechanism of action of Drg1 in the inhibition of apoptosis. Our study suggests that Drg1 plays a critical role in facilitating Bim turnover by mediating its binding to the proteasome. As summarized in Fig. 6, we found that Drg1 mediated the degradation of Bim through the interaction to the ElonginB/Cullin2-CIS ubiquitin-protein ligase complex (28), promoting resistance to chemotherapy. The suppression of Drg1 using siRNA results in Bim stabilization, allowing its association with Hsp70, its mitochondrial localization, and the SN-38-mediated induction of apoptosis. Bim knockdown reversed the effect of SN-38 in Drg1 siRNA-transfected cells, whereas it did not affect the basal level of apoptosis in control cells. BH3-only proapoptotic proteins interact with prosurvival Bcl-2-like proteins inactivating their functions (32). Hence, Bim expression levels have a prominent role in mediating cell death (33).

Previous reports showed that Drg1 is highly expressed in human cancers (14, 15, 17). Looking at the expression levels of Drg1 and Bim in the five cell lines we have tested, it seems that there is a negative correlation between these two proteins. However, more tumor-derived cells need to be analyzed to further confirm and validate the significance of Drg1-mediated regulation of Bim. Recently, it has been reported that RACK1 reduced Bim protein levels in paclitaxel-treated cells through a proteasome-dependent pathway (28). Similarly to RACK1, knockdown of Drg1 by siRNA induced Bim expression. We did not test whether Drg1 differentially regulates Bim in the presence of paclitaxel. However, by immunoprecipitation experiments, we could show that Drg1 binds to a Cullin2/ElonginB-CIS E3 ubiquitin ligase complex in untreated cells. Whereas this novel interaction needs further characterization, we have also shown that Bim binds to the proteasome through Drg1. Conversely, when Drg1 is down-regulated, Bim preferentially bound to Hsp70. In support of this, we could also show that Drg1 competed with Hsp70 for the binding of Bim in vitro (Supplementary Fig. S2). Hsp70 proteins function as chaperons by assisting the folding and assembly of multiprotein complexes, as well as participate in the transport of proteins across cellular membranes (34, 35). We did not find any changes in the expression of Hsp70, whereas its interaction with Bim increased after Drg1 depletion, suggesting a role of Hsp70 in Bim stability and/or localization under these conditions.

Bim also directly bound to the carboxyl-terminal region of Drg1, which may provide the basis to design peptides or small molecules to interrupt this specific interaction. Indeed, finding a way to stabilize and increase Bim levels may provide the means to increase sensitivity to CPT-11–based therapy. In view of the high expression of Drg1 in both primary and metastatic colon cancer to liver, this could result in new treatment paradigms for patients with this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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