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Inhibition of CRD-BP sensitizes melanoma cells to chemotherapeutic agents

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Summary

We previously reported that malignant melanomas express high levels of the mRNA binding protein CRD-BP. This molecule is important for the activation of anti-apoptotic pathways, a mechanism often linked to insensitivity to therapeutics. However, it is not known whether CRD-BP plays a role in the resistance of melanomas to anti-cancer treatment. Here we demonstrate that knock-down of CRD-BP with a specific sh-RNA enhances the effect of dacarbazine, temozolomide, vinblastine and etoposide on both primary and metastatic melanoma cell lines. CRD-BP down-regulation contributes to cell sensitization by increasing apoptosis and diminishing melanoma cell growth in response to chemotherapeutic agents. Furthermore, inhibition of CRD-BP decreases MITF expression and reintroduction of MITF partially compensates for the absence of CRD-BP. These findings suggest that high expression of CRD-BP in melanoma cells confers resistance to chemotherapy and that these CRD-BP responses are mediated, at least in part, by MITF.

Keywords

CRD-BP; MITF; apoptosis; proliferation; drug sensitization

Melanoma is the sixth most common cancer in the United States with an estimated 8700 annual deaths mostly attributed to the aggressive malignant stage (Rubin and Lawrence, 2009, Jemal et al., 2010). However, few medications against metastatic melanoma currently exist and those available exhibit limited efficacy. The first FDA-approved treatment, dacarbazine (DAC), is efficient in only 5–10% of patients while the latest drug, ipilimumab, shows response rates in the 10–20% range (Tarhini and Agarwala, 2006, Poust, 2008). Furthermore, regimens using multiple agents have shown no significant improvement in the rate and/or duration of responses (Poust, 2008). Consequently, increasing melanoma sensitivity to chemotherapeutics remains a vital challenge.

Two major mechanisms thought to be responsible for tumor insensitivity to therapeutics include resistance to apoptosis and the induction of membrane transporters that pump drugs out of the cell (La Porta, 2007). Interestingly, coding region determinant binding protein (CRD-BP) not only increases anti-apoptotic signals but also positively regulates the multidrug resistance transporter MDR-1 (Noubissi et al., 2006, Sparanese and Lee, 2007). Additionally, CRD-BP over-expression has been observed in a variety of human cancers and, in colorectal carcinomas, high levels of CRD-BP are associated with metastasis and poor prognosis (Elcheva et al., 2008, Dimitriadis et al., 2007). These unfavorable conditions occurring in the presence of increased CRD-BP suggest a role for this molecule in tumor

progression. In the present work we document the effect of CRD-BP inhibition on the sensitization of melanoma cells to cytotoxic agents.

First, we investigated how CRD-BP knock-down affects drug-mediated responses by assessing phosphatidylserine (PS) translocation to the outer leaflet, one of the early stages of apoptosis. For this, a specific sh-RNA against *CRD-BP* (sh-*CRD-BP*) was employed in combination with medications commonly used against melanoma: DAC, temozolomide (TMZ), vinblastine (VBN) and etoposide (ETP). These compounds were utilized at concentrations that are within the range of drug plasma levels in cancer patients (Middleton et al., 2000, Brada et al., 1999, Zeffren et al., 1984, Fields et al., 1995). Exposed PS was detected by staining with a fluorophore conjugate of annexin V. Compared to control (scrambled sh-RNA), sh-*CRD-BP* significantly increases annexin V staining in primary and metastatic melanoma cell lines following stimulation with anti-cancer drugs (figures 1A–D and S1–S4). Similar results were obtained with sh-RNA targeting a different region of the CRD-BP transcript (figure S5). On the other hand, melanocytes transfected with control or sh-*CRD-BP* do not exhibit significant changes in annexin V staining following anti-cancer treatment (figures 1E and S6). Hence, CRD-BP inhibition promotes PS translocation in response to chemotherapy in melanoma but not in normal cells. These data demonstrate that CRD-BP down-regulation dramatically increases drug-mediated initiation of apoptosis, thus enhancing the cytotoxic effects of chemotherapeutic agents.

We have recently demonstrated that CRD-BP is highly expressed in melanomas and that microphthalmia-associated transcription factor (MITF) mRNA is a target of CRD-BP (Elcheva et al., 2008, Goswami et al., 2010). These findings were corroborated by real-time PCR experiments showing that CRD-BP is over-expressed in a variety of melanoma cell lines and that sh-*CRDBP* efficiently diminishes CRD-BP and MITF mRNA levels (Figure S7). To further explore the importance of CRD-BP/MITF interactions and the effect of CRD-BP knock-down in chemotherapeutic-induced apoptotic responses, we measured the activity levels of two late apoptosis markers, caspase-3 and caspase-7. As illustrated in figure 2, cells transfected with sh-*CRD-BP* and treated with DAC, TMZ, VBN or ETP (dark gray bars) exhibit a significant increase in caspase activity levels when compared to control (scrambled sh-RNA, black bars). Hence, CRD-BP contributes to melanoma drug insensitivity by ameliorating early and late apoptosis. Next, we studied the role of MITF in these responses by co-transfecting cells with sh-*CRD-BP* and a MITF-expressing construct, followed by stimulation with anti-cancer drugs. Over-expression of MITF decreases caspase activity back to control levels and thus rescues the effects of CRD-BP down-regulation (figure 2, light gray bars). These data support a contribution of MITF in the anti-apoptotic modalities of CRD-BP in melanoma cells.

Both CRD-BP and MITF have been identified as enhancers of melanoma cell growth (Elcheva et al., 2008, Wellbrock et al., 2008). Accordingly, we assessed whether the presence of CRD-BP and MITF is important for melanoma cells to resist the anti-proliferative effects of cytotoxic agents. Although transfection with sh-*CRD-BP* alone decreases colony formation in melanoma cell lines, CRD-BP knockdown potentiates the anti-growth response of melanoma cells to DAC, TMZ and ETP (figure 3). VBN effectively inhibits the formation of colonies at high concentrations (10 ng/mL) (figure 3) but it requires CRD-BP inhibition to completely abrogate colony formation at lower concentrations that are still clinically relevant (1 ng/mL) (figure S8). Similar effects are observed with low levels of TMZ and ETP (figure S8). Thus, down-regulation of CRD-BP increases the efficacy of chemotherapeutics through a synergistic effect.

In cells where CRD-BP is disrupted, ectopic expression of MITF significantly increases colony formation when compared to cells expressing sh-*CRD-BP* alone (figures 3 and S8).

However, the addition of MITF does not restore the ability of cells to form colonies back to control levels. These results suggest that CRD-BP regulates cell proliferation partly through MITF, although other mechanisms may be involved. One possible candidate also participating in this response is nuclear factor kappa B (NF- κ B), a transcription factor with anti-apoptotic and proliferative functions that is up-regulated in melanomas (Dror et al., 2010). CRD-BP induces NF- κ B by stabilizing β TrCP1 which results in fast turnover of inhibitory kappa B (I κ B) and subsequent translocation of NF- κ B to the nucleus (Noubissi et al., 2006). Here, we have found that NF- κ B activity is highly increased in melanoma cells when compared to melanocytes (figure S9-A, black bars). Furthermore, CRD-BP inhibition significantly decreases the activation of NF- κ B in melanomas but not in melanocytes (figure S9-A, gray bars). Other likely players include MDR-1 and c-myc, which are known direct targets of CRD-BP (Sparanese and Lee, 2007). Initial experiments show that MDR-1 and c-myc are over-expressed in melanoma cells and that knock-down of CRD-BP significantly diminishes MDR-1 and c-myc levels in these cells (figure S9-B and S9-C). Thus, down-regulation of NF- κ B, MDR-1 and c-myc by CRD-BP knock-down may also participate in melanoma drug sensitization. Future studies will establish a role for NF- κ B, MDR-1 and c-myc in relaying CRD-BP signals during melanoma cell resistance to chemotherapy.

In summary, we have demonstrated that CRD-BP plays a relevant role in melanoma cell resistance to therapy by disrupting apoptosis and promoting cell growth. In this context, MITF is an important downstream target, as it mediates a significant part of CRD-BP-induced cellular responses. Nevertheless, it is likely that CRD-BP exerts its effects through several other pathways. Thus, CRD-BP may act as a master regulator of signaling mechanisms that work in concert to render melanoma cells insensitive to chemotherapeutics. Knock-down of CRD-BP noticeably enhances the response to four medications already used for the treatment of melanoma. Consequently, inhibition of CRD-BP could prove to be a novel strategy to increase the efficacy of currently available anti-cancer agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Despite the recent development of novel therapies, malignant melanomas remain notoriously resistant to drugs, with less than 25% of patients obtaining durable responses with currently available treatments. Thus, the identification of new approaches to circumvent melanoma resistance is of the utmost importance. Here we show that CRD-BP expression correlates with increased MITF levels in melanoma and that CRD-BP signals partly through MITF to facilitate cell resistance to chemotherapy. Inhibition of CRD-BP decreases MITF levels and enhances the pro-apoptotic and anti-proliferative effects of chemotherapeutic agents. Therefore, targeted disruption of CRD-BP may be a valuable tool to improve current drug efficacy.

Figure 1.

Effect of CRD-BP inhibition and chemotherapeutic treatment on annexin V positivity. (A–E) Cells transfected with GFP and either scrambled sh-RNA or sh-*CRD-BP* were treated with DAC (10 μ g/mL), TMZ (100 μ M), VBN (10 ng/mL) or ETP (100 ng/mL) for 18 hrs. Only cells expressing GFP and showing binding of Annexin V were counted as Annexin V positive. The data are shown as the mean $\pm$ SD of cells counted in five different fields. * $P < 0.01$, compared to scrambled sh-RNA (control).

Figure 2.

The role of CRD-BP and MITF in caspase activation by chemotherapeutic agents. (A–D) Cells transfected with scrambled sh-RNA, sh-*CRD-BP* or sh-*CRD-BP* + MITF were treated with DAC (10 μ g/mL), TMZ (100 μ M), VBN (10 ng/mL) or ETP (100 ng/mL) for 48 hrs. Caspase 3/7 activity was determined using the Caspase Glo 3/7 assay (Promega, Madison, WI). The data are representative of 4 independent experiments performed in duplicate and are expressed as mean values $\pm$ SD. * $P < 0.05$, compared to scrambled sh-RNA (control).

Figure 3.

Effect of CRD-BP knock down and MITF overexpression on the regulation of cell proliferation by anti-cancer drugs. The indicated cell lines were transfected with pTk-puro and either scrambled sh-RNA (control), sh-*CRD-BP* alone or sh-*CRD-BP* + MITF. Cells were selected for puromycin resistance and treated with DAC (10 μ g/mL), TMZ (100 μ M), VBN (10 ng/mL) or ETP (100 ng/mL) for 12 days. The resulting colonies were fixed, stained with crystal violet and counted. The data are representative of 3 independent experiments performed in duplicate and are expressed as mean values $\pm$ SD. * $P < 0.05$, compared to untreated cells transfected with sh-*CRD-BP*.