Chemistry & Biology
Article

The Natural Anticancer Compounds Rocaglamides Inhibit the Raf-MEK-ERK Pathway by Targeting Prohibitin 1 and 2

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http://dx.doi.org/10.1016/j.chembiol.2012.07.012

SUMMARY

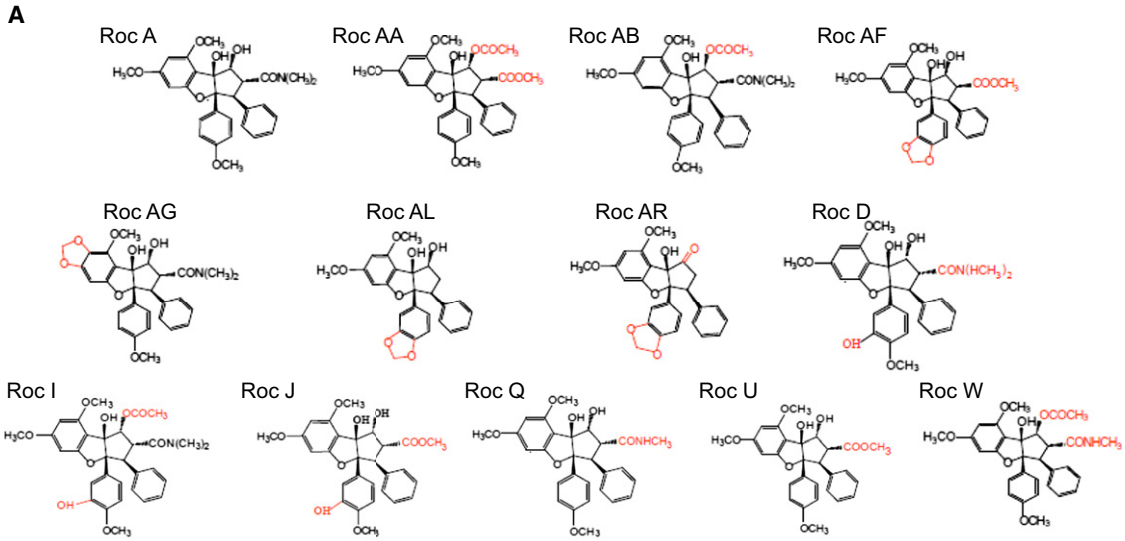
Rocaglamides are potent natural anticancer products that inhibit proliferation of various cancer cells at nanomolar concentrations. We have recently shown that these compounds prevent tumor growth and sensitize resistant cancer cells to apoptosis by blocking the MEK-ERK-eIF4 pathway. However, their direct molecular target(s) remain(s) unknown. In this study, using an affinity chromatography approach we discovered that prohibitin (PHB) 1 and 2 are the direct targets of rocaglamides. Binding of rocaglamides to PHB prevents interaction between PHB and CRaf and, thereby, inhibits CRaf activation and subsequently CRaf-MEK-ERK signaling. Moreover, knockdown of PHB mimicked the effects of rocaglamides on the CRaf-MEK-ERK pathway and cell cycle progression. Thus, our finding suggests that rocaglamides are a new type of anticancer agent and that they may serve as a small-molecular tool for studying PHB-mediated cellular processes.

INTRODUCTION

During the past decades, a group of phytochemicals collectively named rocaglamides (= flavaglines) isolated from the genus *Aglaia* (family Meliaceae) have been found to possess anticancer activities tested in various tumor cell lines as well as in patient samples in vitro (Kim et al., 2006; Ebada et al., 2011; Zhu et al., 2007) and to inhibit tumor growth in vivo in several mouse tumor models (McPhail et al., 1982; Emerson et al., 2010; Lucas et al., 2009). *Aglaia* plants consist of approximately 130 species distributed mainly in the tropical rain forests of Southeast Asia from Sri Lanka, India, Vietnam, to south China. Several species of this genus are traditionally used in folk medicine for treatment of coughs, injuries, asthma, and inflammatory skin diseases. The extracts from the *Aglaia* species are also traditionally used as an insect repellent (Kim et al., 2006; Ebada et al., 2011).

The first bioactive molecule of *Aglaia* extracts, rocaglamide (thereafter refers to Roc-A) (Figure 1A), was identified in 1982, and was shown to increase the lifespan of tumor-bearing mice in a leukemia model (McPhail et al., 1982). To date, more than 100 naturally occurring rocaglamide-type compounds, characterized by a cyclopenta[*b*]-tetrahydrobenzofuran backbone, have been isolated from over 30 *Aglaia* species and many of them have been shown to inhibit tumor growth at nM concentrations (Kim et al., 2006; Ebada et al., 2011). The primary effect of rocaglamides on tumor growth inhibition was shown to be due to inhibition of protein synthesis without affecting synthesis of DNA and RNA (Ohse et al., 1996; Lee et al., 1998).

Although rocaglamides were found to inhibit translation 15 years ago, their mode of action remained largely unknown. Translation is initiated by binding of the initiation factor eIF4E to the mRNA 5′ cap structure. After binding to the 5′ cap structure, eIF4E interacts with eIF4G, which serves as a scaffold protein for the assembly of eIF4E and eIF4A to form the eIF4F complex. The eIF4F complex is then directed to the 5′ terminus of the mRNA, unwinds the mRNA 5′ secondary structure to facilitate ribosome binding, and promotes ribosome recruitment and translation (Silvera et al., 2010). Recently, the rocaglamide derivative silvestrol was shown to inhibit translation by stimulation of the RNA-binding properties of eIF4A. This action prevents incorporation of free eIF4A into the eIF4F complex (Cencic et al., 2009). We have recently demonstrated that several rocaglamides inhibit the mitogen and extracellular-signal regulated protein kinase kinase (MEK) and extracellular-signal regulated protein kinase (ERK) signaling (Zhu et al., 2007; Bleumink et al., 2011). The Ras-mediated MEK-ERK pathway is one of the key signaling transduction pathways that regulate protein synthesis and tumor survival (Balmanno and Cook, 2009; Silvera et al., 2010). We have shown that rocaglamides do not directly inhibit the translational machinery, but they suppress MEK-ERK signaling, leading to inhibition of phosphorylation of eIF4E, the key translation initiation factor that controls the rate-limiting step of cap-dependent translation (Bleumink et al., 2011;



B

Rocaglamides		A	AA	AB	AF	AG	AL	AR	D	I	J	Q	U	W	FL3
Effect on proliferation [48 h] IC_{50} ± s.e.m. [nM]	DMSO	8.1 ± 0.8	>1000	6.9 ± 1.3	367.9 ± 28.4	15.9 ± 2.7	35.6 ± 4.5	15.3 ± 2.0	13.5 ± 1.1	78.4 ± 13.9	12.5 ± 4.2	14.2 ± 1.8	26.4 ± 4.6	43.0 ± 9.7	6.2 ± 1.4
Effect of Rocs [100 nM] on ERK [16 h] pERK1/2 (%)	100	8.01	84.33	15.78	94.86	22.50	41.35	13.82	19.27	73.63	24.32	23.00	32.07	19.82	0.00

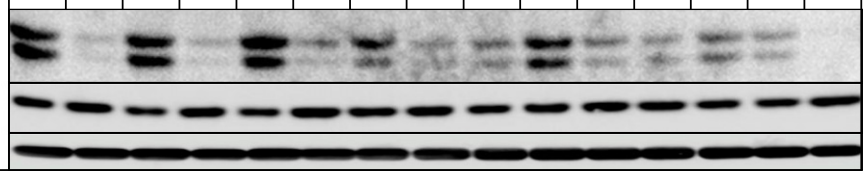


Figure 1. Rocaglamides-Mediated Inhibition of Proliferation Correlates with Downregulation of ERK Activity

(A) Chemical structures of 13 natural occurring rocaglamides.

(B) Correlation between proliferation inhibition and suppression of ERK activity. Jurkat cells were treated with different concentrations of rocaglamides. After 16 hr treatment, samples were taken for examination of the activation status of ERK by western blot. Levels of phosphorylated ERK (pERK1/2) were quantified using the BIO-1D software (Vilber Lourmat, Marne-la-Vallée, France). The level of pERK1/2 in DMSO-treated cells is taken as 100%. Cell proliferation rates were determined after 48 hr treatment by the Cell Titer Glo assay. Concentrations that cause 50% of inhibition (IC_{50}) of cell proliferation are shown. Data are an average of four independent assays.

Blagden and Willis, 2011). Consequently, this action does not only prevent tumor cell growth but also leads to sensitization to extrinsically induced apoptosis in resistant cancer cells by downregulation of antiapoptotic proteins (Bleumink et al., 2011; Giaisi et al., 2012). However, the direct molecular target(s) of rocaglamides in the ERK signaling pathway remain unknown.

In this study, we carried out an affinity chromatography approach using rocaglamide-conjugated affinity beads to identify potential rocaglamide-interacting proteins. Using this strategy, two rocaglamide-binding proteins, prohibitin 1 (PHB1) and prohibitin 2 (PHB2), were identified. PHBs are evolutionarily conserved and ubiquitously expressed proteins that have been implicated in regulation of diverse cellular processes, such as mitochondrial biogenesis and function, cell signaling, apoptosis, survival, and proliferation (Rajalingam and Rudel, 2005; Osman et al., 2009; Merkwirth and Langer, 2009; Theiss and Sitaraman, 2011). Recently, PHB1 was shown to be involved in regulation of the Ras-mediated Raf-MEK-ERK signaling pathway (Rajalingam et al., 2005), which plays an important role in regulation of survival and proliferation in a broad range of human tumors (Balmanno and Cook, 2009; Silvera et al., 2010). In this study, we show that rocaglamides bind to both PHB1 and PHB2 and inhibit their interaction with CRaf. This event leads to inhibition of PHB/CRaf-mediated activation of the MEK-ERK signaling pathway and, consequently, inhibits protein synthesis, cell cycle progression, and cell proliferation in malignant cells.

RESULTS

Rocaglamide-Mediated Inhibition of Proliferation Correlates with Downregulation of ERK Activity

We have previously shown that several rocaglamide derivatives suppress the MEK-ERK signaling pathway (Zhu et al., 2007; Bleumink et al., 2011). To confirm that the antiproliferative effect of rocaglamides is indeed due to a suppression of the ERK pathway, we examined 14 rocaglamide derivatives including

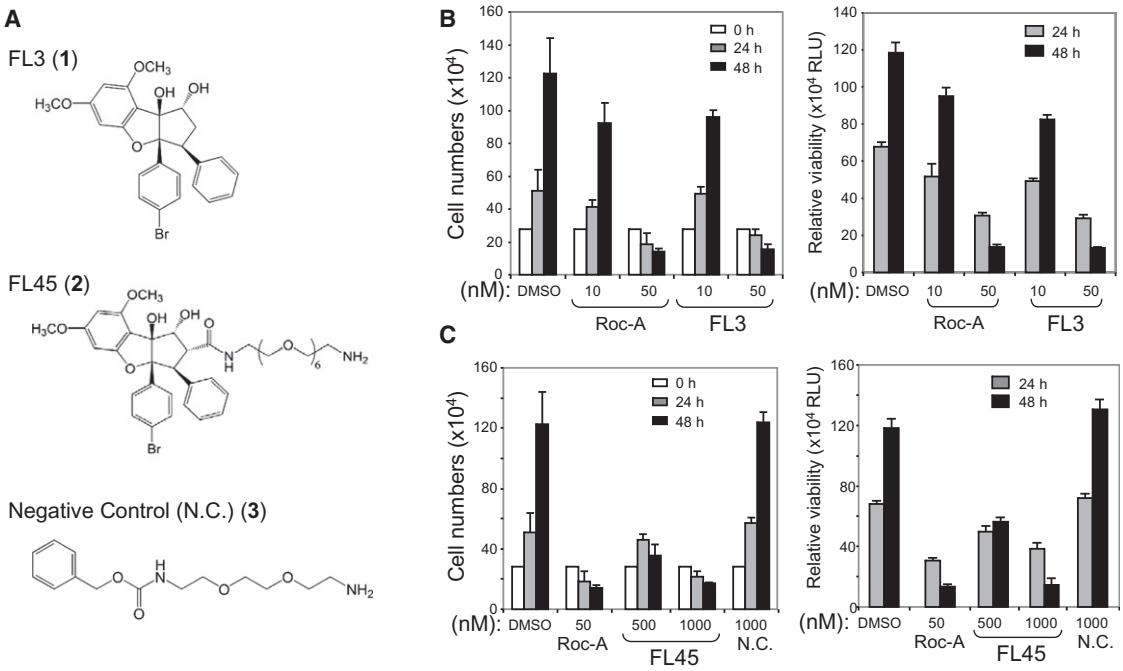


Figure 2. Rocaglamides Used for Identification of Potential Cellular Targets

(A) Chemical structures of synthetic rocaglamide derivative FL3 (1), linker-coupled FL45 (2), and NC (3) compounds used in this study.

(B) Effects of Roc-A and FL3 on cell proliferation in cancer cells. Jurkat leukemic cells were incubated with either Roc-A or FL3 for indicated periods. Cell proliferation was determined by counting the cell numbers (left panel) and by the Cell Titer Glo assay (right panel). Data are representative of two independent experiments. Means ± SD are shown.

(C) Effects of FL45 and NC on cell proliferation in cancer cells. Jurkat cells were incubated with FL45 and NC, followed by examination of cell proliferation as in (B). Data are representative of two independent experiments. Means ± SD are shown.

the synthetic FL3 (Thuaud et al., 2009) for their effects on ERK activity and proliferation (Figures 1A and 2A). The experiment showed a significant correlation between IC_{50} values for inhibition of proliferation and the phosphorylation status of ERK (Figure 1B). For instance, Roc-AA and Roc-AF did not inhibit cell proliferation and showed no or very little effect on ERK activity. Rocaglamides with lower IC_{50} concentrations for proliferation inhibition showed stronger suppression of the ERK activities. These data provide evidence that rocaglamides inhibit cell proliferation by blocking the ERK signaling pathway.

Identification of Prohibitins as Cellular Targets of Rocaglamide

To investigate the cellular targets of rocaglamides, we first attempted to identify rocaglamide-binding proteins by affinity chromatography using rocaglamide-conjugate-coupled Affi-Gel beads. Therefore, we conjugated the biologically active derivative FL3 (Thuaud et al., 2009) to a molecular linker to generate the derivative FL45 (Figure 2A), which was subsequently coupled to the Affi-Gel beads. Although FL45 showed a lesser efficacy compared to Roc-A and FL3, it still maintains growth inhibitory activity at the nM concentration (Figures 2B and 2C). A benzyl carbamate of the linker, used as a negative control (NC), was shown to have no inhibitory activity on proliferation (Figures 2A and 2C). Total cell extracts from Jurkat cells were used for the affinity chromatography experiment and two proteins of approximately 32 and 37 kDa were specifically purified on the FL45 beads (Figure 3A). Mass spectrometry (MS) analysis revealed that the FL45-binding proteins are PHB1 and PHB2. This was confirmed by western blot analysis using antibodies against PHB1 and PHB2, respectively (Figure 3B).

To confirm that rocaglamides bind to PHB1 and PHB2, the affinity chromatography experiment was repeated with Flag-tagged purified PHB1 and PHB2 proteins. The experiment showed that recombinant Flag-PHB1 and Flag-PHB2 proteins bind to FL45 but not to the NC beads (Figure 3C). Specificity of the interaction between PHBs and FL45 was further examined by adding free Roc-A or Roc-AA as competitors in the affinity chromatography assay. As expected, Roc-A prevented binding of PHB1 to FL45, whereas Roc-AA had no effect on binding of PHB1 to FL45 (Figure 3D). These data demonstrate that PHB1 and PHB2 are the cellular targets of rocaglamides.

Roc-A Inhibits the Interaction between PHB and CRaf

It has been shown that CRaf (= Raf-1) activation requires a direct interaction with the PHB1 protein (Rajalingam et al., 2005). Therefore, we further investigated the effect of rocaglamides on interactions between PHB1 and CRaf. Jurkat cells were treated with either Roc-A or its vehicle (DMSO) for 3 hr. Total cell lysates were subjected to immunoprecipitation by anti-CRaf antibodies followed by western blot with antibodies against either PHB1 or CRaf. Consistent with the previous report (Rajalingam et al., 2005), CRaf was coprecipitated with PHB1. However, in the presence of Roc-A the interaction between PHB1 and CRaf was

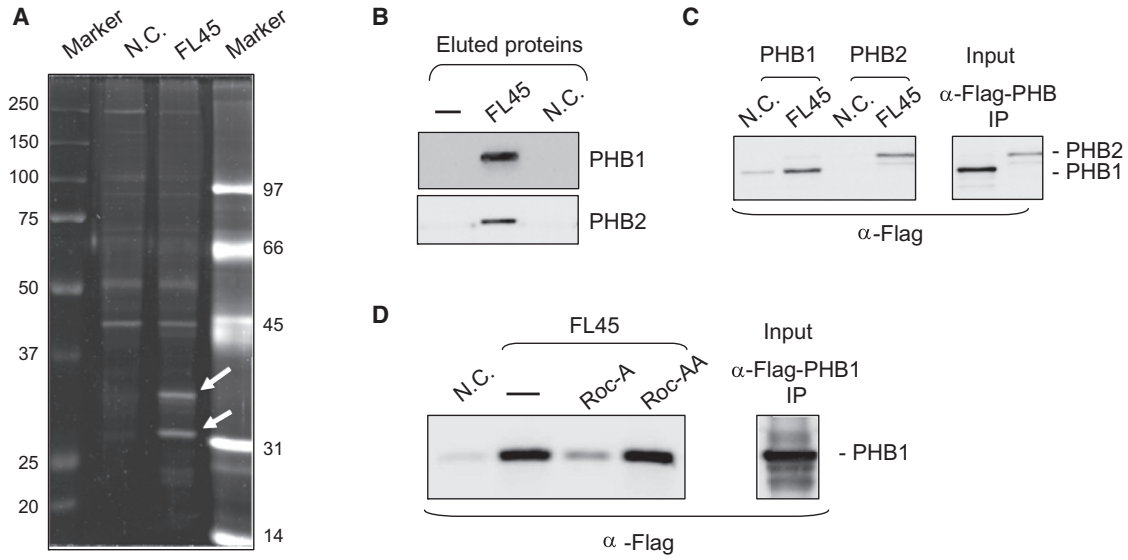


Figure 3. PHB1 and PHB2 Are Direct Targets of Rocaglamides

(A) Purification of rocaglamides-binding proteins by affinity chromatography with FL45-conjugated beads. FL45-conjugated or NC beads were incubated with total cell lysates from Jurkat cells. The bound proteins were eluted and subjected to SDS-PAGE, visualized by Flamingo Pink, and processed for mass spectrometry as described in the Experimental Procedures section. MS analyses revealed the two major bands at 32 and 37 kDa (indicated by arrows) being PHB1 and PHB2, respectively.

(B) The eluted proteins were subjected to western blot with PHB1 and PHB2 antibodies. Data are representative of three independent experiments.

(C) Purified Flag-PHB proteins were used in the affinity chromatography experiment. FL45 or NC beads were incubated with purified Flag-PHB1 and Flag-PHB2 proteins. The bound proteins were eluted and were subjected to western blot using Flag antibody. The input of Flag-precipitated PHB1 and PHB2 is shown on the right panel.

(D) The experiment in (C) was carried out with Flag-PHB1 in the absence or presence of 50 nM of Roc-A or Roc-AA.

significantly diminished (Figure 4A). To confirm this observation, we repeated the experiment using HEK293T cells transiently transfected with Flag-PHB1 and Flag-PHB2 expressing plasmids in the absence or presence of Roc-A or Roc-AA. At the cellular level, PHB1 has been shown to localize to the cell membrane and the mitochondrial inner membrane together with PHB2 (Nijtmans et al., 2000; Sharma and Qadri, 2004; Merkwirth and Langer, 2009). The experiment showed that both Flag-PHB1 and Flag-PHB2 were coprecipitated with CRaf. The coprecipitation was abolished by Roc-A, but not by Roc-AA (Figure 4B).

To further confirm above observations, recombinant Flag-PHB1 and CRaf proteins were mixed in the absence or presence of Roc-A. PHB1 was immunoprecipitated with a Flag antibody followed by western blot with Flag and CRaf antibodies. This experiment clearly showed that in the presence of Roc-A, CRaf was no longer coprecipitated with PHB1 (Figure 4C). The same result was obtained using recombinant Flag-PHB2 protein. As shown in Figure 4D, Roc-A, but not Roc-AA prevented PHB2 interacting with CRaf (Figure 4D). These data demonstrate that rocaglamides prevent the interaction of both PHB1 and PHB2 with CRaf.

Recently, the marine product aurilide was shown to bind PHB1, but not PHB2 in the mitochondria, and this interaction resulted in mitochondrial fragmentation after 4 hr and apoptotic cell death after 16 hr in HeLa cells (Sato et al., 2011). Therefore, we also examined the effect of rocaglamides on the mitochondrial morphology and apoptosis induction in Jurkat and HeLa cells. In contrast to aurilide, Roc-A did not cause mitochondrial fragmentation in both cell types (Figure 4E) and did not induce apoptosis in HeLa cells (data not shown). In Jurkat cells, a moderate apoptosis induction (less than 20%) was observed after 36 hr treatment with Roc-A (Zhu et al., 2007). The ineffectiveness of rocaglamides on the mitochondrial morphology cannot be explained by an inability of rocaglamides to reach the mitochondria as fluorescently labeled FL3 was seen to be co-localized with mitochondria (Figure 4F).

Rocaglamides Inhibit the Raf-MEK-ERK Signaling Pathway

Since Roc-A inhibits the interaction of PHB with CRaf, we predicted that rocaglamides may block CRaf activation. To examine this prediction, Jurkat cells were treated with or without Roc-A for different periods, and the phosphorylation status of CRaf at the activating phosphorylation site Ser338 was analyzed by western blot. As expected, Roc-A inhibited CRaf phosphorylation in a time-dependent manner and blocked phosphorylation of the kinases MEK and ERK downstream of CRaf (Figure 5A). In comparison, Roc-A had no effect on activities of ARaf and BRaf (Figure 5A).

To further confirm that Roc-A-mediated inhibition of CRaf activation is due to inhibition of interactions between PHB and CRaf, we performed a knockdown experiment using specific PHB siRNAs. Consistent with the above experiment, knockdown of PHB1 or PHB2 could mimic the inhibitory effect of Roc-A on CRaf (but not ARaf and BRaf) and the Raf-MEK-ERK pathway (Figure 5B). Consistent with other studies (Merkwirth et al.,

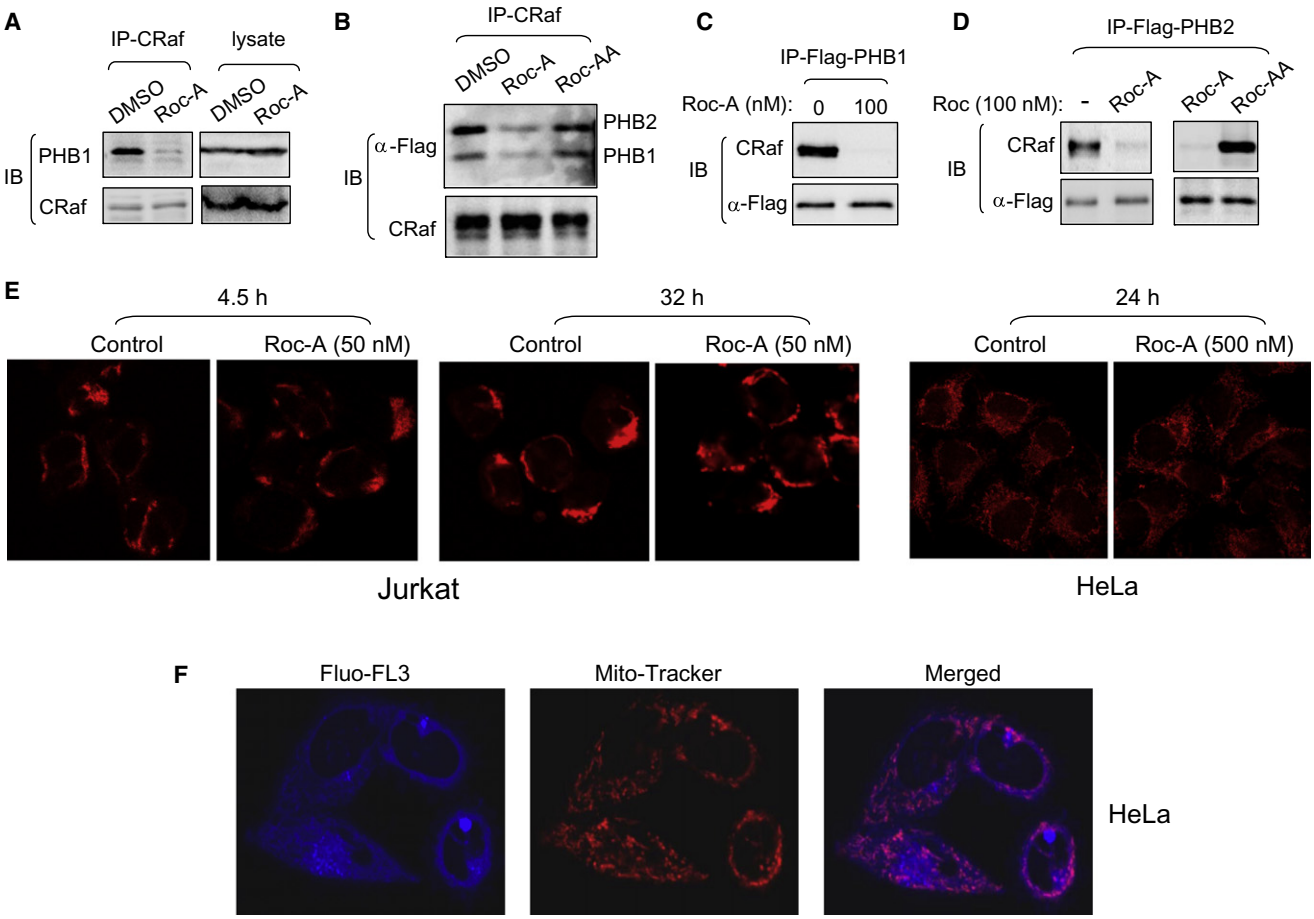


Figure 4. Roc-A Inhibits Interaction between PHB and CRaf

(A) Roc-A inhibits binding of CRaf to PHB in vivo in Jurkat cells. Jurkat cells were incubated either with Roc-A (50 nM) or solvent DMSO for 3 hr, and CRaf was immunoprecipitated followed by western blot analysis with PHB1 and CRaf antibodies. Data are representative of two independent experiments.

(B) Roc-A, but not Roc-AA, inhibits binding of CRaf to PHB1/2 in vivo in HEK293T cells. HEK293T cells were transiently transfected with plasmids expressing Flag-PHB1 and Flag-PHB2. After 24 hr transfection, the cells were treated with solvent DMSO or Roc-A (50 nM) or Roc-AA (50 nM) for 4 hr. CRaf was immunoprecipitated followed by western blot analysis with Flag and CRaf antibodies.

(C) Roc-A inhibits the interaction of PHB1 with CRaf in vitro. Recombinant CRaf protein was incubated with purified Flag-PHB1 protein in the absence or presence of Roc-A (100 nM). PHB1 was immunoprecipitated with a Flag antibody followed by western blot using antibodies against CRaf and Flag. Data are representative of three independent experiments.

(D) Roc-A inhibits the interaction of PHB2 with CRaf in vitro. The experiment was carried out as described in (C) using recombinant Flag-PHB2 in the presence of either Roc-A or Roc-AA.

(E) Roc-A has no effects on the mitochondrial morphology in Jurkat and HeLa cells. Jurkat and HeLa cells were treated with 50 nM (for Jurkat) and 500 nM (for HeLa) of Roc-A for indicated periods. Mitochondria were stained by Mito Tracker Red.

(F) Localization of rocaglamides in mitochondria. HeLa cells were treated with fluorescently labeled FL3 (Fluo-FL3, blue) (50 μM). Mitochondria were stained by Mito Tracker Red.

2008; Sievers et al., 2010), a specific knockdown of either PHB1 or PHB2 at the mRNA level (data not shown) resulted in downregulation of both proteins. This indicates that PHB1 and PHB2 need each other for protein stabilization.

To exclude the possibility that rocaglamides might directly inhibit Raf activity, we carried out an in vitro assay by incubation of active Raf with recombinant MEK protein in the absence or presence of different concentrations of Roc-A. The experiment showed that all three isoforms of Raf phosphorylated MEK. However, Roc-A could not block Raf-mediated activation of MEK (Figure 5C). Thus, rocaglamides do not directly inhibit Raf activity.

To further investigate whether rocaglamides inhibit the CRaf-MEK-ERK signaling pathway upstream of CRaf, we tested the effect of rocaglamides on ERK activation stimulated by phorbol myristate acetate (PMA), which is known to indirectly activate CRaf via activation of protein kinase C (PKC) (Kolch et al., 1993). The experiment showed that Roc-A failed to inhibit ERK activity induced by PMA (Figure 5D). Furthermore, we showed that knockdown of PHB proteins resulted in inhibition of translation similar to the effect of Roc-A (Figures 5E and 5F). These data support that rocaglamides inhibit the Raf-MEK-ERK signaling pathway by interfering with the interaction between PHB and CRaf.

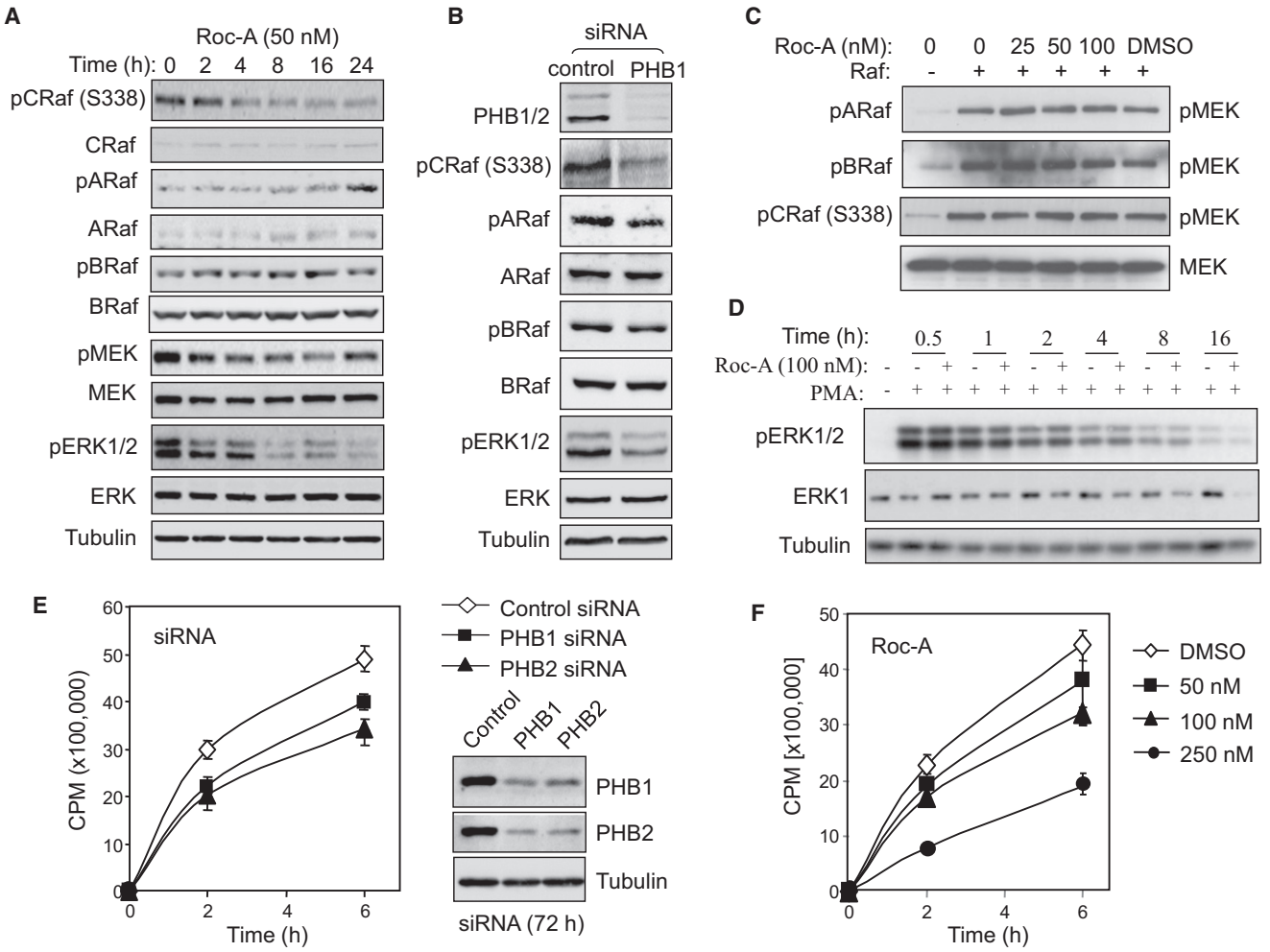


Figure 5. Knockdown of PHB Mimics the Effect of Roc-A on the CRaf-MEK-ERK Pathway

(A) Roc-A inhibits the CRaf-MEK-ERK signaling pathway. Jurkat cells were treated with Roc-A (50 nM) for different time periods as indicated. Cell lysates were subjected to western blot with antibodies against total and phosphorylated ARaf, BRaf, CRaf (Ser338), MEK, and ERK. Data are representative of three independent experiments.

(B) Knockdown of PHB mimics the effect of Roc-A on the CRaf-MEK-ERK pathway. Jurkat cells were transfected with either control or siRNA specific for PHB1. Sixty hr after transfection, cells were subjected to western blot analysis using the indicated antibodies. Data are representative of two independent experiments.

(C) Roc-A does not directly inhibit activity of Raf. Activated Raf proteins were incubated with purified MEK protein in the absence or presence of different concentrations of Roc-A as indicated. The status of phosphorylated MEK was determined by western blot with MEK and phospho-MEK antibodies as indicated. Data are representative of two independent experiments.

(D) Roc-A does not inhibit PMA-induced activation of ERK. Jurkat cells were stimulated with PMA (5 ng/ml) in the presence or absence of Roc-A (100 nM) for indicated periods. The activities of ERK were examined by western blot with phospho-ERK antibody as indicated. ERK1 and tubulin antibodies were used for equal loading control. Data are representative of three independent experiments.

(E) siRNA knockdown of PHB inhibits protein synthesis in malignant cells. Jurkat cells were transfected with control or PHB siRNA. After 72 hr transfection, the rates of protein synthesis were determined by ^{35}S-methionine incorporation. The efficacies of knockdown were controlled by western blot. Data are representative of two independent experiments assayed in triplicates.

(F) Roc-A inhibits protein synthesis in malignant cells. Jurkat cells were treated with different concentration of Roc-A. The rates of protein synthesis were determined as in (E). Data are representative of two independent experiments assayed in triplicates.

Knockdown of PHB Mimics the Effect of Rocaglamides on Cell Cycle Progression

To further investigate the role of PHB in regulation of cell proliferation, the effects of Roc-A and PHB siRNA on the cell cycle were assayed in Jurkat cells. Treatment of Jurkat cells with Roc-A arrested cell cycle at the G0/G1 phase (Figures 6A and 6B). In addition, the expression levels of proteins important for the G1-S transition, such as cyclin D3, CDK4, CDK6, and cdc25A (Boutros et al., 2007; Malumbres and Barbacid, 2009), were strongly downregulated by Roc-A (Figure 6C). In contrast, the expression levels of cdc25C and CDK1, which are mainly involved in driving the transition from S to G2/M phase (Boutros et al., 2007; Malumbres and Barbacid, 2009), were unchanged (Figure 6C). Similar to the effect of Roc-A, knockdown of PHB1 and PHB2 in Jurkat cells by siRNA led to inhibition of cell cycle progression at the G1/S phase via downregulation of the G1/S

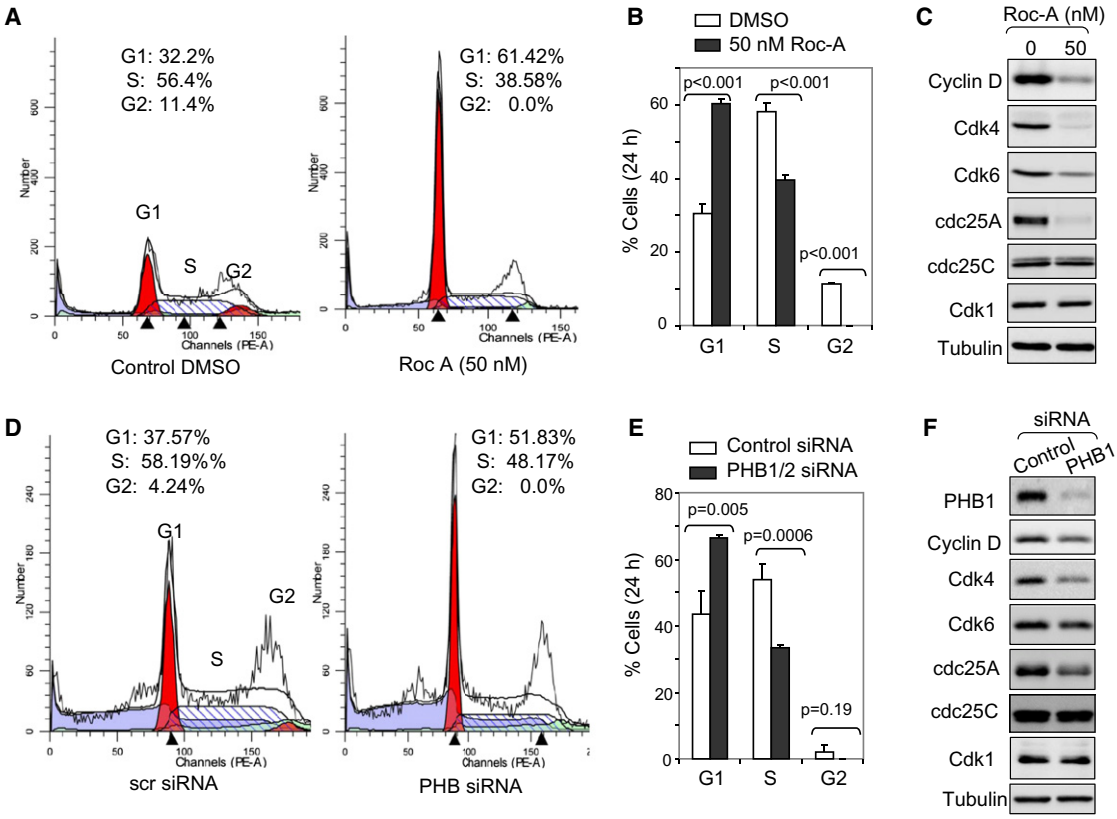


Figure 6. siRNA Knockdown of PHB Mimics the Effect of Roc-A on Cell Cycle Progression

(A) Roc-A induces cell cycle arrest at the G0/G1 phase accompanied with reduction in S phase in Jurkat cells. Jurkat cells were treated with 50 nM of Roc-A or DMSO as indicated. Cell cycle distributions were determined after 24 hr treatment.

(B) Data from (A) are presented by bar charts. The results are representative of three independent experiments. Means ± SD are shown.

(C) Roc-A downregulates expression of G1/S phase regulatory proteins in Jurkat cells. The cells from (A) were subjected to western blot for examination of the expression levels of proteins controlling the G1/S phase. Data are representative of three independent experiments.

(D) siRNA knockdown of PHB results in blocking of cell cycle progression similar to Roc-A. Jurkat cells were transfected with PHB1/2 siRNAs. After 24 hr of transfection, the status of cell cycle was determined as in (A).

(E) Data from (D) are presented by bar charts. The data are representative of two independent experiments. Means ± SD are shown.

(F) siRNA knockdown of PHB results in reduced expression of G1/S regulatory proteins. The data are representative of two independent experiments.

See also Figure S1.

regulatory proteins cyclin D3, CDK4, CDK6, and cdc25A (Figures 6D–6F). Thus, knockdown of PHB proteins can mimic the effect of Roc-A on cell cycle progression.

Above data suggest that rocaglamides exert their antiproliferative effect by interfering with PHB function. Along this line, overexpression of PHB proteins would be expected to render the cells more resistant to rocaglamide. To study this prediction, Jurkat cells were transiently transfected with either the Flag-control or the Flag-PHB1 and Flag-PHB2 expression plasmids and the effects of Roc-A on proliferation of the transfected cells were examined. The experiment showed that Jurkat cells transfected with Flag-PHB1/2 expression plasmids proliferated faster than the cells transfected with the control plasmid (Figure S1A available online). As predicted, overexpression of Flag-PHB1/2 rendered Jurkat cells more resistant to Roc-A (Figure S1B). However, the Flag-PHB-mediated resistance was not as strong as expected. This might be due to the difference between Flag-PHB and the wild-type PHB proteins.

Rocaglamides Prevent PHB-CRaf Membrane Association

PHB was previously shown to be required for membrane association and activation of CRaf (Rajalingam et al., 2005). Therefore, we asked whether rocaglamides affect PHB-CRaf membrane association. To investigate this question, cell membrane and cytosol fractions were prepared from Jurkat cells treated with either Roc-A or Roc-AA to analyze the localization of PHB and CRaf. Western blot analysis showed a significant reduction in CRaf, particularly phosphorylated CRaf (pSer338), in the membrane fraction after Roc-A treatment (Figure 7A). Interestingly, Roc-A also significantly reduced the levels of PHB in the membrane fraction indicating that binding of rocaglamides to PHB may also interfere with PHB membrane association. However, Roc-A did not influence Ras membrane localization (Figure 7A). These results further demonstrate that rocaglamides block the CRaf-MEK-ERK signaling pathway by interfering with the PHB function (Figure 7B).

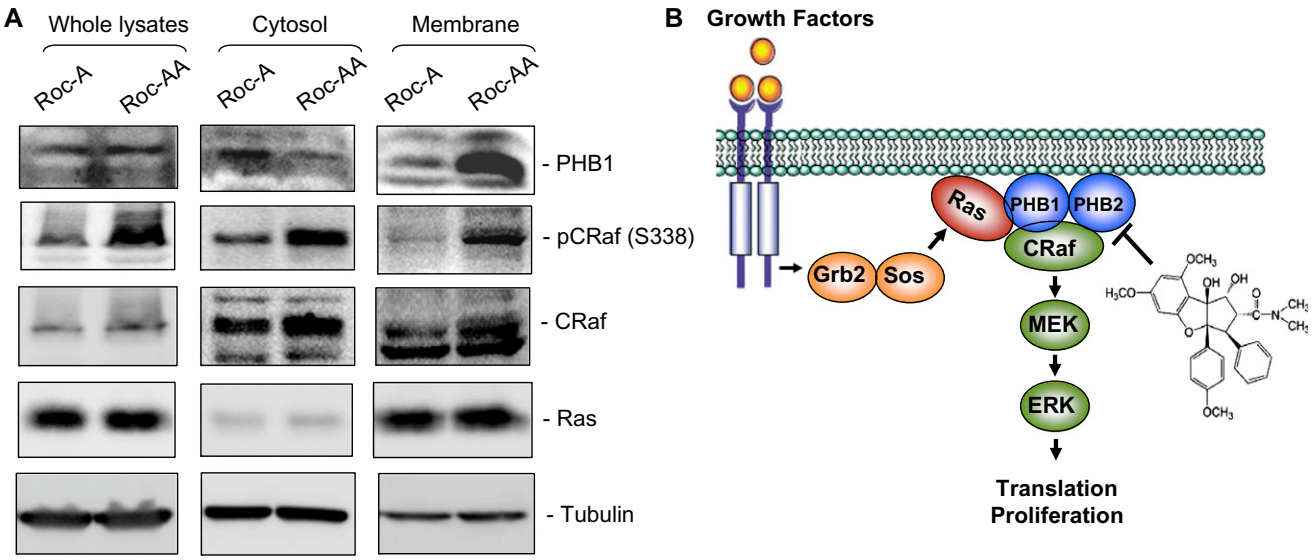


Figure 7. Roc-A Prevents PHB-CRaf Membrane Localization

(A) Analysis of PHB-CRaf membrane localization in Jurkat T cells in the presence of Roc-A or Roc-AA. Jurkat cells were treated with 100 nM of Roc-A or Roc-AA for 16 hr. Whole-cell lysates and fractions of cell membrane and cytosol were prepared and were subjected to western blot analysis with antibodies as indicated. Phospho-CRaf (Ser338) was used as marker of activity and tubulin as loading control. Results are representative of two independent experiments.

(B) Schematic representation of the mechanism by which rocaglamides inhibit the Raf-MEK-ERK pathway.

DISCUSSION

We previously found that ERK activity is downregulated by different rocaglamide derivatives in malignant cells, but not in normal cells (Zhu et al., 2007). Recently, we demonstrated that rocaglamides inhibit protein synthesis primarily via blocking the MEK-ERK-Mnk-eIF4E signaling pathway (Bleumink et al., 2011). However, the direct molecular targets of rocaglamides in the ERK signaling pathway were unknown.

In the present study, using affinity chromatography, we show that rocaglamides bind directly to PHB1 and PHB2. This is further confirmed by using purified recombinant PHB proteins in the affinity chromatography assay (Figure 3). PHB1 was previously shown to interact with CRaf, and this interaction was necessary for CRaf activation by Ras (Rajalingam et al., 2005). Consistent with the previous study, we confirm that PHB1 interacts with CRaf and demonstrate that binding of rocaglamides to PHB1 proteins prevents interaction between PHB1 and CRaf (Figures 4A–4D). In addition, we show that PHB2 also binds to CRaf and that this interaction is blocked by rocaglamides, too (Figures 4B and 4D). We further show that binding of rocaglamides to PHB proteins or targeting PHB by siRNAs inhibits CRaf phosphorylation and consequently suppresses translation, cell cycle progression, and cell proliferation in malignant cells (Figures 5 and 6). Furthermore, we show that rocaglamides do not only inhibit interaction of PHB and CRaf, but also interfere with PHB and CRaf membrane association (Figure 7A). Taken together, our study reveals the molecular mechanism of the anticancer action of rocaglamides (Figure 7B).

Activation of the Ras-Raf-MEK-ERK pathway has been shown to be associated with proliferation in a broad range of human tumors (Maurer et al., 2011; Balmanno and Cook, 2009; Silvera et al., 2010), which makes this pathway an important molecular target of anticancer therapy (Sebolt-Leopold and English, 2006; Roberts and Der, 2007). Recently, treatment with small-molecule inhibitors of BRaf has been shown to yield unprecedented response rates of 70%–80% as single agents in melanoma patients with $BRaf^{V600E}$ mutation (Bollag et al., 2010; Flaherty et al., 2010; Kefford et al., 2010). However, selective inhibition of BRaf has been found to drive Ras-dependent activation of CRaf and MEK-ERK signaling leading to drug resistance and even to drug-mediated tumor progression (Heidorn et al., 2010; Halaban et al., 2010; Kwong and Chin, 2010). We show that rocaglamides specifically inhibit CRaf activation by preventing interaction between PHB and CRaf. So far, small molecules specifically inhibiting CRaf activity have not been reported. Thus, rocaglamides may be used in combination with BRaf inhibitors to enhance efficacies of cancer therapy and to overcome drug-resistance. We also show that rocaglamides do not directly inhibit Raf activity. Instead, rocaglamides inhibit CRaf activation by Ras via preventing the interaction of CRaf and PHB. This suggests that rocaglamides are new types of inhibitors of the Raf-MEK-ERK signaling pathway and may serve as a novel anticancer agent.

PHB has previously been reported to play a regulatory role in the PI3K-Akt signaling pathway via interaction with phosphatidylinositol 3,4,5-triphosphate and Akt (Héron-Milhavet et al., 2008; Ande and Mishra, 2009). The PI3K-Akt signaling pathway is inappropriately activated in many cancers, which promotes protein synthesis and proliferation (Engelman, 2009). The activity of the translation initiation factor eIF4E is also regulated by the PI3K-AKT-mTOR pathway (Silvera et al., 2010). We have recently demonstrated that rocaglamides do not inhibit mTOR phosphorylation (Bleumink et al., 2011). Thus, rocaglamides inhibit protein synthesis primarily via blocking the MEK-ERK-Mnk-eIF4E signaling pathway.

PHBs are ubiquitous, evolutionarily conserved proteins. The first mammalian PHB (PHB1) was identified in 1989 as a potential tumor suppressor with antiproliferative activity and was hence named prohibitin (McClung et al., 1989). However, the antiproliferative activity was found to be attributed to the 3′-UTR of the PHB1 mRNA, and it is unrelated to the function of the protein itself (Jupe et al., 1996). The role of PHB1 in cancer proliferation and/or tumor suppression has been controversially discussed. Increased PHB1 levels have been reported in cancers of cervix, esophagus, stomach, breast, lung, bladder, thyroid, ovary, and prostate, indicating its potential role in cancer progression (Theiss and Sitaraman, 2011). However, opposing results have also been observed, e.g., decreased PHB1 levels in gliomas and somatic mutations in the PHB1 gene in a few sporadic breast cancers (Theiss and Sitaraman, 2011). Our data show that rocaglamides inhibit CRaf activation and, consequently, cell proliferation in leukemic cells. In addition, knockdown of PHB via siRNA resulted in inhibition of cell cycle progression of Jurkat cells. Thus, our study supports an essential role of PHB in regulation of cancer proliferation.

Recently, the marine product aurilide was shown to selectively bind to PHB1 in the mitochondria leading to rapid (after 4 hr) mitochondrial fragmentation and apoptosis induction after 16 hr in HeLa cells (Sato et al., 2011). In contrast, rocaglamides bind both PHB1 and PHB2, and this interaction does not target mitochondria for degradation in both Jurkat and HeLa cells (Figure 4D). Since PHBs also regulate cell cycle progression, transcriptional regulation and cellular signaling in other cellular compartments depending on the cell type (Mishra et al., 2005, 2010; Artal-Sanz and Tavernarakis, 2009), our finding of direct binding of rocaglamides to PHBs also suggests that these compounds may serve as a new molecular probe for studying PHB-mediated cellular processes.

SIGNIFICANCE

To improve cancer treatment, there is an urgent need to better understand signaling in cancer cells and to develop new anticancer drugs. Although the ability of the natural compounds rocaglamides to inhibit tumor cell proliferation was discovered more than 15 years ago, their mode of action has been elusive. We have recently shown that rocaglamides suppress the MEK-ERK-Mnk1 signaling pathway, which is one of the key pathways that regulate protein synthesis, proliferation, and tumor survival. However, the direct target(s) remained unknown. In this study, we found that the direct molecular targets of rocaglamides are PHB1 and PHB2. Binding of rocaglamides to PHBs prevents their interaction with CRaf and, thereby, inhibits CRaf activation and Raf-MEK-ERK-mediated cell cycle progression and cell proliferation in cancer cells. So far, small molecules specifically inhibiting CRaf activity have not been reported. Thus, rocaglamides may serve as a new type of anticancer agent. The role of PHB1 in cancer proliferation and/or tumor suppression has been controversially discussed because both overexpression and reduced expression or deletion/mutations in the PHB1 gene have been reported in human tumors. We show that targeting PHB1 or 2 via siRNA mimics the inhibitory action of rocaglamides on CRaf activation and proliferation. Thus, our study supports the important role of PHBs in regulation of cancer proliferation. Furthermore, as PHB proteins are involved in regulation of several important signaling pathways in different cellular compartments, our finding also suggests that rocaglamides may serve as a new small-molecular tool for studying PHB-mediated cellular processes.

EXPERIMENTAL PROCEDURES

Rocaglamide Derivatives

Roc-A (rocaglamide) (>98% pure) was purchased from Enzo Life Sciences (Lörrach, Germany). FL3 and fluorescently labeled FL3 was described previously (Thuaud et al., 2009). The other rocaglamide derivatives used in this study are Roc-AA (C-1-O-acetyl-methylrocaglate) (Chaidir et al., 1999); Roc-AB (1-*O*-acetyl-rocaglamide) (Hiort et al., 1999); Roc-AF (3′,4′-methylendioxy-methylrocaglate) (Cui et al., 1997); Roc-AG (aglaroxin A) (Dreyer et al., 2001; Molleyres et al., 1999); Roc-AL (4′-demethoxy-3′,4′-methylenedioxyrocaglaol) (Cui et al., 1997); Roc-AR (1-oxo-4′-demethoxy-3′,4′-methylenedioxyrocaglaol) (Cui et al., 1997); Roc-D (C-3′-hydroxyrocaglamide) (Nugroho et al., 1997); Roc-I (C-1-O-acetyl-3′-hydroxy-rocaglamide) (Nugroho et al., 1997); Roc-J (3′-hydroxyaglafoline) (Nugroho et al., 1999); Roc-Q (demethylrocaglamide) (Ishibashi et al., 1993; Dumontet et al., 1996); Roc-U (methylrocaglate, aglafoline) (Ishibashi et al., 1993; Dumontet et al., 1996); and Roc-W (C-1-*O*-acetyl-desmethylrocaglamide) (Hiort et al., 1999). All compounds had been isolated previously from various *Aglaia* species (see above cited literature) and are >98% pure assessed by high-performance liquid chromatography (HPLC).

Cell Lines and Culture

The human T cell leukemic cell line Jurkat, the human embryonic kidney cell line HEK293T, and the human cervical cancer cell line HeLa were used in this study. Cells were cultured in Roswell Park Memorial Institute 1640 or Dulbecco's modified Eagle's medium, respectively, and supplemented with 10% fetal calf serum, 100 U/ml penicillin (Gibco, Invitrogen, Darmstadt, Germany), 100 μg/ml streptomycin (Gibco, Invitrogen), and 2 mM L-glutamine (Gibco, Invitrogen) at 37°C and 5% CO_2.

Cell Proliferation Assay

Cells were treated with different doses of rocaglamides (solved in DMSO) for indicated times. Cell proliferation was determined by counting the cell numbers and by using the Cell Titer Glo cell viability assay kit according to the manufacturer's instructions (Promega, Mannheim, Germany).

Western Blot Analysis and Immunoprecipitation

For western blot analysis, 1×10^6 cells were lysed as previously described (Zhu et al., 2007). Equal amounts of proteins were separated on 5%–13% SDS-PAGE depending on the molecular sizes of the proteins and blotted onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfon, UK) as previously described (Zhu et al., 2007). Subcellular fractionations were carried out as described previously (Kamiński et al., 2012). The following antibodies were used: anti-PHB1 and anti-PHB2 were kindly provided by V. Bosch; anti-CDK2 was kindly provided by I. Hoffmann; antibodies against Ras, ARaf, BRaf, CRaf, MEK, PHB1, phospho-ARaf (S299), phospho-BRaf (S445), phospho-CRaf (S338), phospho-MEK, cdc25C (5H9), CDK4, CDK6, and Cyclin D3 were purchased from Cell Signaling Technology (Danvers, MA, USA); cdc25A Ab3 (Clone DCS-120 + DCS-121) from Neomarkers (Thermo Scientific, Cornwall, UK); CDK1 from Exbio (Prague, Czech Republic); ERK1 from BD Biosciences (Erembodegem, Belgium); FLAG and Tubulin from Sigma-Aldrich (St. Louis, USA); and pERK from Santa Cruz Biotechnology (Heidelberg, Germany).

For immunoprecipitation, cells were washed in ice-cold PBS and lysed in buffer containing 10 mM Tris-HCl pH 7.5, 300 mM NaCl, 1% NP-40, and 10% glycerin and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Lysates were cleared by centrifugation for 30 min at 10,000 g. After addition of an equal volume of buffer containing 10 mM Tris-HCl pH 7.5, 1% NP-40, and 10% glycerin and protease inhibitor cocktail, supernatants were incubated with indicated antibodies and sepharose-coupled protein A beads overnight.

Generation of FL45-Affi-Gel

To identify rocaglamide-interacting proteins, affinity chromatography was performed using Affi-Gel-10 agarose beads (Bio-Rad Laboratories, Hercules, CA, USA) covalently coupled to the chemically synthesized rocaglamide derivative FL45 (the coordinate to the compound 6 is described in Thuaud et al., 2009) or to NC, an inactive derivate. Coupling of the compound FL45 to Affi-Gel-10 was performed according to the manufacturer's instructions. Briefly, 500 μl of packed Affi-Gel-10 were washed with isopropanol and dimethylformamide (DMF). Subsequently, 400 μl of 12 mM FL45 or NC dissolved in DMF supplemented with 2.5% (v/v) triethylamine were added. This suspension was gently rotated at room temperature (RT) for 12 hr. After blocking of any remaining active ester groups using aminopropanol for 4 hr, the matrix was thoroughly washed with DMF, ethanol, and H_2O. Coupled Affi-Gel-10 agarose beads were kept in PBS supplemented with 0.02% NaN_3 at 4°C until further use.

Affinity Chromatography

For affinity chromatography, 1 × 10^8 Jurkat cells were washed in PBS and lysed in 2 ml lysis buffer containing 50 mM Tris/HCl pH 8.0, 120 mM NaCl, 1% NP-40, 5 mM DTT, 200 μM Na-orthovanadate, 25 mM NaF, and protease inhibitor cocktail (Roche Diagnostics). Cellular debris was removed by centrifugation at 10,000 g for 30 min. 500 μg of total protein extract was incubated for 12 hr at 4°C with 40 μl of FL45, NC-coupled or uncoupled Affi-Gel beads. The beads were extensively washed with lysis buffer, and bound proteins were eluted and reduced in a sample buffer containing 63 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, a trace bromophenol blue, and 200 mM DTT for 30 min at 65°C. After cooling on ice, the sample was alkylated with a final concentration of 150 mM iodacetamide for additional 30 min. Proteins were separated by SDS-PAGE (10% gel) in Protean II electrophoresis units (Bio-Rad Laboratories) and visualized by Flamingo Pink (Bio-Rad Laboratories) following the manufacturer's instructions. Two major protein bands at approximately 32 and 37 kDa were detected by fluorescence scanning using a Typhoon Trio Laser Scanner (GE Healthcare, Munich, Germany) in FL45, but not control samples (Figure 3A). Nevertheless, same sections of the gel were picked in the NC to ensure a specific identification in the FL45 eluents. These bands were picked automatically using an Ettan spotpicker (GE Healthcare), manually processed for MS by tryptic digestion, and spotted onto steel grids as described elsewhere (Schmidt et al., 2008).

MS Analysis

The samples were analyzed by peptide mass fingerprinting using the 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA, USA). For MS analyses, typically 1,000 shots were accumulated in positive reflector mode for each spot. In addition, MS/MS analysis was used for validation of the probability mass function. Therefore, precursor ions were selected automatically according to the following criteria: a maximum of five masses per spot and a signal-to-noise ratio >35. The precursor mass window was set to 50 (full width at half maximum). All peptide mass spectra were processed by internal calibration with autolytic fragments of porcine trypsin with 25 ppm mass tolerance using the GPS Explorer software version 3.6 (Applied Biosystems). Mass spectrometric data were searched against the NCBI database with a taxonomy restriction to human proteins (216,738 entries, November 11, 2009) using MASCOT V2.0 (Matrix Sciences, London).

Analysis of Mitochondrial Morphology

For Jurkat cells, 1 × 10^6 cells were treated with or without Roc-A for indicated periods and then stained for 15 min with 50 nM of MitoTracker Deep Red FM (Invitrogen). Cells were washed twice with PBS, fixed with 3% paraformaldehyde for 10 min at 37°C in the dark, and permeabilized with 90% methanol for a minimum of 30 min at 4°C or left overnight at −20°C. The fixed and permeabilized cells were washed three times, transferred to microscopy slides, and the mitochondrial morphology was examined at the Zeiss LSM710 microscope (Zeiss, Jena, Germany). For HeLa cells, cells were grown on microscopy chamber slides (Lab-Tek, Thermo Scientific) and treated with Roc-A and mitoTracker as described in Jurkat cells. After washing twice with PBS, cells were fixed with 4% paraformaldehyde for 15 min at RT in the dark. After additional washing steps, cells were subjected to permeabilization with 0.2% Triton X-100 for 10 min at RT in the dark. After washing, cells were examined at the Zeiss LSM710 microscope. The fluorescently labeled FL3 was described previously (Thuaud et al., 2009).

Knockdown Experiment

Jurkat cells (2 × 10^6) were transfected in Nucleofector solution (Nucleofector kit V, Amaxa Biosystems, Cologne, Germany) with 1–2 μM of nonsense siRNA (QIAGEN, Hilden, Germany) or siRNAs specific for PHB1 (5′-CAGAAAUCACU GUGAAAUUTT-3′ [QIAGEN]) or PHB2 (5′-CCCAGGAAUUCUCAAUAAATT-3′ [QIAGEN]) using the Amaxa Nucleofector apparatus and the program X-01. Cells were collected at indicated time points after transfection for cell cycle measurement, translation assays, and protein expression analysis.

Plasmids and Transfections

Expression plasmids for Flag-PHB1 and Flag-PHB2 have been described and were kindly provided by V. Bosch (Emerson et al., 2010). As Flag-control plasmid PP4R1 tagged with Flag was used. The expression plasmids were transfected into HEK293T cells using the standard calcium phosphate method. Flag-PHB1 and Flag-PHB2 proteins were purified from HEK293T by immunoprecipitation using a Flag antibody and Flag-peptide elution. For Jurkat cells, transfection was carried out with Nucleofector solution (Nucleofector kit V, Amaxa Biosystems).

In Vitro Binding Assay

For in vitro binding assays, purified Flag-PHB1 or Flag-PHB2 protein was incubated with recombinant CRaf protein in the presence or absence of 100 nM Roc-A for 2 hr. Subsequently, PHB1 or PHB2 was immunoprecipitated using a Flag antibody. The recombinant human CRaf (RAF1) expressed by baculovirus in Sf9 insect cells using an N-terminal glutathione S-transferase tag (GTX65339) was purchased from GeneTex (Irvine, CA, USA). The gene accession number is NM_002880. The purity was determined to be >85% by densitometry.

In Vitro Kinase Assay

To obtain activated Raf, Jurkat T cells were stimulated with PMA for 2 hr, and the cells were subjected to immunoprecipitation with antibodies against different Raf proteins. To examine the effect of rocaglamides on Raf activity in vitro, immunoprecipitated Raf was incubated with or without Roc-A for 10 min, and then recombinant MEK proteins (Upstate Biotechnology, Lake Placid, NY, USA) were added and further incubated for 30 min at 30°C. The status of activated MEK was examined by western blot with antibodies against MEK and pMEK.

Cell Cycle Determination

For cell cycle analysis, approximately 1 × 10^6 cells were collected, lysed in 150 μl of Nicoletti-buffer (0.1% Na-citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide), and stored at 4°C overnight in the dark. The propidium iodide stained DNA fragments were quantified by flow cytometry (FACSCanto II, BD Biosciences). The ModFit LT program was used for the cell cycle analysis.

Translation Assay

Protein synthesis was estimated by measuring the amount of incorporated ^{35}S-methionine. Briefly, cells (1 × 10^6) were incubated for 3 hr in methionine-free medium. Then 7 μCi per well of ^{35}S-methionine-labeling mix (PerkinElmer, Waltham, MA, USA) were added. After 2–6 hr incubation, cells were washed with PBS and lysed in ice-cold lysis buffer for 15 min on ice and centrifuged (10 min, 13,000 rpm). Then, 50 μl of each lysate was incubated in 1 ml of Liquid Scintillation Cocktail solution (Beckman Coulter, Brea, CA, USA), and radioactivity was determined with Liquid Scintillation counting.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.07.012.

ACKNOWLEDGMENTS

This work was supported in part by the Helmholtz Alliance on Immunotherapy of Cancer in the Helmholtz association, the "Association pour la Recherche sur le Cancer", and Conectus. We thank Melanie Nebendahl for technical assistance.

Received: March 6, 2012
Revised: June 12, 2012
Accepted: July 10, 2012
Published: September 20, 2012

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