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13. ABSTRACT (Maximum 200 Words)

DAMD17-03-1-0317 is an IDEA award that focuses on the roles of BCCIP in cell cycle regulation. The approaches are over-expression and down-regulation of BCCIP by RNAi. The project has been moving on smoothly in the first 12 months. We have accomplished the scientific objectives of tasks 1-3, and 50% of tasks 4-5. We have published one paper based on this study. As an un-anticipated discovery, we have found that BCCIP also regulates p21 expression. This finding further strengthens our central hypothesis that BCCIP regulates cell cycle control. In the next funding year, we will strive to accomplish the objectives of tasks 5-6.

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Regulation of Cell Cycle by BCCIP, a BRCA2 and CDKN1A (Cip1/p21) Interacting Protein

Introduction

DAMD17-03-1-0317 is an IDEA award that focuses on the roles of BCCIP in cell cycle regulation. The approaches are over-expression and down-regulation of BCCIP by RNAi. The project has been moving on smoothly in the first 12 months of the award. We have begun gaining significant insight on the roles of BCCIP in cell cycle control.

Body of Report

Tasks 1-3 are related to specific aim 1. It involves the construction of an inducible BCCIPβ expression system (task 1), and determination of its effects on cell growth (task 2) and cell cycle progression (task 3). The original plan was to use MCF7 cells but HT1080 was proposed as an alternative. Cell growth inhibition by BCCIP has been measured in MCF7 and several other cell lines as planned. However, we noticed that cell cycle measurement was more reproducible in HT1080 cells than MCF7 cells. Thus, we have moved on to use the alternative HT1080 cell line when cell cycle measurement is involved. The results have been included in a publication (see appendix 1 for details). The main conclusions from this study were that over-expression of BCCIP inhibits cell growth (including MCF7 cells) (Figures 2 and 3 of Appendix 1), and delays the progression of G1 to S transition (Figure 5 in Appendix 1). However, we did not observe any significant effect of BCCIP overexpression on G2/M transition. Therefore, we consider the objectives of tasks 1-3 have been achieved.

One unanticipated result from tasks 1-3 was that p21 expression can be induced by BCCIP expression (Figure 6 of Appendix 1). This is a significant novel finding, since it implicates that BCCIP may regulate p21 function by at least two pathways, i.e. by regulating p21 expression, and by directly interacting with p21.

Tasks 4 and 5 are to determine the genetic interactions between p21, BRCA2 and BCCIP genes in cell cycle control by over-expressing BCCIP in BRCA2 and p21 deficient cells. We have found that over-expression of BCCIP resulted in reduced cell growth inhibition in a p21 deficient background (Figure 4 of Appendix 1). Because deficiency of p21 itself results in cell cycle deregulation, we did not observe further defect in G1/S checkpoint even BCCIP was over-expressed. Therefore, we consider the objectives on genetic interaction between p21 and BCCIP have been accomplished. We will concentrate on the genetic interaction between BRCA2 and BCCIP in the context of cell cycle control in the future.

The finding that over-expression of BCCIP induces p21 levels (tasks 1-3) has led us to investigate whether down-regulation of BCCIP results in reduced p21 expression. Because BRCA2 regulates p53 activity and p21 expression, we further hypothesize that BCCIP may regulate p21 expression by a BRCA2 dependent pathway. This will serve as an ideal platform to accomplish the rest objectives of tasks 4-5, i.e. the genetic interaction between BCCIP and BRCA2 in regulating cell cycle. This will be our focus in the next 12 month.

Tasks 6-7 will establish cell lines with impaired BCCIP function to determine the role of BCCIP in cell cycle. We have initiated experiments using RNAi interference to achieve this goal. We have made retrovirus and adenovirus based short hairpin RNA expression systems to achieve the

objectives of tasks 6-7. We anticipate reporting the results of these studies in the next annual report.

Task 8: we have published 1 manuscript (appendix 1). We anticipate that at least one manuscript will be submitted/published in the next 12 months.

Key Research Accomplishments

- We have confirmed that BCCIPβ also interacts with p21
- Using an inducible system, we have demonstrated that overexpression of BCCIP delays G1 to S transition
- We have shown a p21-dependent growth inhibition by over-expressed BCCIP
- We have found a potential novel mechanism by which BCCIP may regulate p21 function, i.e. via the regulation of p21 expression

Reportable Outcome

One publication (attached as appendix 1):
Xiangbing Meng, Jingmei Liu, and Zhiyuan Shen, Inhibition of G1 to S Cell Cycle Progression by BCCIPβ. Cell Cycle, 2004, 3:343

Conclusions

The major scientific findings in the last 12 months are: BCCIP regulates p21 expression and inhibits G1 to S progression. This suggests a critical role of BCCIP in cell cycle control. We have accomplished the objectives of tasks 1-3, and 50% of the tasks 4-5. RNA silencing vectors to be used for tasks 6-7 have been constructed.

References: none

Appendix

1. Xiangbing Meng, Jingmei Liu, Zhiyuan Shen, 2004. Inhibition of G1 to S Cell Cycle Progression by BCCIPβ. Cell Cycle, 3:343

[Cell Cycle 3:3, 343-348; March 2004]; ©2004 Landes Bioscience

APPENDIX 1

Report

Inhibition of G_1 to S Cell Cycle Progression by BCCIPβ

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KEY WORDS

BCCIP, Tok-1, p21, CDKN1A, Cip1, G_1-S transition, BRCA2

ABBREVIATIONS

BCCIP	BRCA2 and CDKN1A (Cip1/p21/Waf1) Interacting Protein
Doc	doxcycline
Noc	nocodazole
WCE	whole cell extract

ACKNOWLEDGMENTS

We thank Dr. Bert Vogelstein (Johns Hopkins University) for providing HCT116p21$^{-/-}$(8054) and the HCT116p21$^{+/+}$ parental cell, and Mr. Adrian Maestas for critical reading of the manuscript.

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ABSTRACT

The BCCIPα protein was identified as a BRCA2 and CDKN1A (p21, or p21$^{Waf1/Cip1}$) Interacting Protein. It binds to a highly conserved domain proximate to the C-terminus of BRCA2 protein and the C-terminal domain of the CDK-inhibitor p21. Previous reports showed that BCCIPα enhances the inhibitory activity of p21 toward CDK2 and that BCCIPα inhibits the growth of certain tumor cells. Here we show that a second isoform, BCCIPβ, also binds to p21 and inhibits cell growth. The growth inhibition by BCCIPβ can be partially abrogated in p21 deficient cells. Overexpression of BCCIPβ delays the G_1 to S progression and results in an elevated p21 expression. These data suggest BCCIPβ as a new regulator for the G_1-S cell cycle progression and cell growth control.

INTRODUCTION

The human BCCIPα (also named Tok-1α) was independently identified by two groups as a BRCA2 and p21$^{Waf1/Cip1}$ (referred as p21 hereafter) interacting protein.[1,2] The BCCIPα interacting domain of BRCA2 (amino acids 2973-3053) resides in a recently termed OB2 domain according to the BRCA2 crystal structure.[1,3] The BCCIPα interacting domain of p21 locates at the C-terminus overlapping the PCNA binding domain and the AKT phosphorylation region of p21.[2,4-6] Overexpression of BCCIPα inhibits cell growth[1] and enhances the inhibitory activity of p21 toward the histone H1 kinase activity of CDK2, suggesting a role for BCCIPα in G_1/S cell cycle control.[2] A second isoform, BCCIPβ, has also been identified.[1,2] These two isoforms, BCCIPα (322 amino acids) and BCCIPβ (314 amino acids), are products of alternative splicing of a single gene on chromosome 10q26, and they share 258 identical N-terminal amino acids.[7] In addition, certain cancer tissues and cell lines display reduced expression of BCCIPα and BCCIPβ.[1,7] However, it was not clear whether or not BCCIPβ also plays a role in G_1/S control.

Ono et al mapped the p21 interacting region to an internal BCCIPα domain that is shared with BCCIPβ,[2] which would suggest an interaction between BCCIPβ and p21. Yet, the same report was not able to demonstrate this interaction using an in vitro protein binding assay.[2] In addition, it was not known whether BCCIPβ would inhibit cell growth as BCCIPα did. The purpose of this report is to clarify these issues. We show that BCCIPβ also interacted with p21 in vivo and inhibited cell growth. We further show that BCCIPβ delayed the G_1-S progression during the cell cycle. In addition, the cell growth inhibition effects of BCCIPα and BCCIPβ was partially abrogated by p21 defect, and overexpression of BCCIPα or BCCIPβ stimulated p21 expression. These data suggest BCCIPβ as a new regulator for G_1-S progression.

MATERIAL AND METHODS

Cell Culture and Gene Transfection. HT1080 cells were cultured in α-MEM medium (Gibco BRL, Grand Island, NY). HeLa, MCF7, 293, A172, HCT116/p21$^{-/-}$(8054) and HCT116/p21$^{+/+}$ cells were cultured in DMEM medium (Hyclone, Logan, UT). All cell culture medium were supplemented with 10% fetal calf serum (Nova-Tech Inc., Grand Island, NE) and 1% Penicillin/Streptomycin (Biowhittaker, Walkersville, MD). Plasmid DNA transfection into mammalian cells was performed with the Geneporter transfection kit (Gene Therapy Systems Inc., San Diego, CA), and retroviral mediated gene transfer was performed as previously described.[1]

Doxcycline (Doc)-Inducible BCCIPβ Expression. Doc-inducible overexpression of BCCIPβ in HT1080 cells was established by pRevTet-ON system (Clontech, Palo Alto, CA). HA-tagged BCCIPβ were cloned into pREV-TRE (Clontech, Palo Alto, CA) via the SalI site while under the control of the tetracycline-responsive element (TRE), resulting in pREV-TRE/HA-BCCIPβ. The latter vector was introduced via retroviral infections into HT1080 cells that had been previously

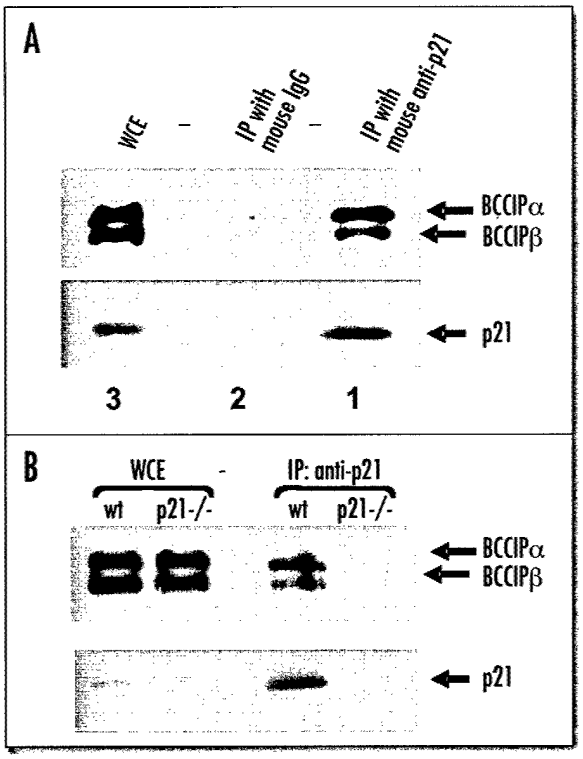

Figure 1. Interaction between BCCIP and p21. (A) Equal amounts of whole cell extract (WCE) from HCT116 cells were incubated with agarose resin conjugated with mouse anti-p21 monoclonal antibody to precipitate endogenous p21 protein (lane 1). Nonspecific mouse IgG conjugated to the same resin was used as the negative control (lane 2). Lane 3 is the whole cell extract. The top panel was blotted with rabbit anti-BCCIP antibodies, and the bottom panel was blotted with anti-p21 antibody. The figure shows that endogenous BCCIPαβ proteins can be coprecipitated with p21. (B) Whole cell extract from p21 wild type (lanes wt) and p21 deficient (lanes $p21^{-/-}$) HCT116 cells were precipitated by anti-p21 monocloncal antibody conjugated with agarose resin. The coprecipitated endogenous BCCIP proteins were visualized by anti-BCCIP blot (top panel). The precipitated p21 was visualized by anti-p21 blot (bottom panel). This figure shows that BCCIPαβ can only be coprecipitated with the anti-p21 resin from the p21 wild type cells, but not the p21 deficient cells.

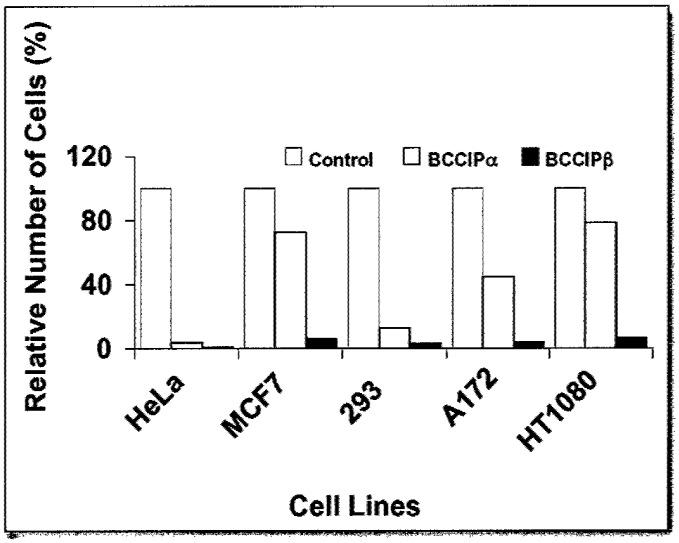

Figure 2. Inhibition of cell growth by retroviral-mediated BCCIP expression HeLa, MCF7, 293, A172 and HT1080 cells were plated at 0.1 x 10^6 cells/ml. Eighteen hours after plating, the cells were infected with viruses that expressed HA-BCCIPα and HA-BCCIPβ. Virus packed with vacant vector was used as control. Infected cells were selected with puromycin for 96 hours and allowed to grow for an additional 3 days. The numbers of cells were then determined by counting. Shown are the relative numbers of cells normalized to the cells infected with vacant control vectors.

transfected with the pTet-On vector that expressed rtTA transcription factor. Selection in neomycin and hygromycin resulted in a Doc-inducible HA-BCCIPβ expressing cell line designated HT1080/TetOn-BCCIPβ. Doc-induced HA-BCCIPβ expression in this cell line was confirmed by anti-HA immunoblot.

DNA Content Based Cell Cycle Analysis. The method originally described by Vindelov et al was adapted to stain nuclear DNA for flow cytometry analysis.[8] Briefly, cells were trypsinized, washed with PBS, and fixed with 70% ethanol. Then cells were pelleted, washed with PBS, and resuspended in 200 µl of citrate buffer (250 mM Sucrose, 0.05% DMSO, 40 mM Trisodium citrate, pH7.6). Nine hundred microliters of Solution A [0.003% trypsin in stock buffer (3.4 mM Trisodium citrate, 0.1% Nonidet P 40, 1.5 mM Spermine tetrahydrochloride, 0.5 mM Trizma, pH 7.6)] were added and cells were incubated at room temperature for 10 minutes. Afterwards, 750 µl of Solution B (0.025% Trypsin inhibitor, 0.01% Ribonuclease A in stock buffer) was added for another incubation of 10 minutes at room temperature. Then, 750 µl of Solution C (0.0416% Propidium iodide, 3.3 mM Spermine tetrahydorchloride in stock buffer) was added to the cells, which were then ready for flow cytometry analysis. In each assay, 20,000 cells were collected by FACScan (Becton Dickinson, San Jose, CA) and analyzed by CellQuest software provided.

Antibodies and Western Blot. Cells were treated in lysis buffer (50 mM HEPES pH7.6, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40). Proteins were separated by 10% SDS-PAGE. BCCIP or p21 were detected by appropriate specific antibodies. Anti-p21 antibodies were purchased from BD Transduction Laboratories (Lexington, KY). The anti-BCCIP antibodies have been described previously.[1] In certain cases, β-actin monoclonal antibody was used to confirm that equal amount of protein quantity was loaded in each samples.

Immunoprecipitations. To precipitate the endogenous p21 protein, the Ab3 mouse anti-p21 monoclonal antibody (Oncogene Research Products™, La Jolla, CA) was immobilized to protein G agarose beads by Seize™X Mammalian Immunoprecipitation Kit (Pierce, Rockford, IL). Equal amount of nonspecific mouse IgG conjugated agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was used as negative control. Cells were washed with PBS, pelleted, treated with lysis buffer (50 mM Tris-HCl PH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.25% NP-40, 1 mM Na_3VO_4, 100 µg/ml PMSF, 20 µg/ml aprotinin, 10 µg/ml Leupeptin.), sonicated, and centrifuged for 10 min at 15,000 rpm. The supernatant (whole cell extract) was treated with Benzonase nuclease (Novagene, Madison, WI), which digests DNA and RNA to eliminate their potential interference in protein complex formation. One mg whole cell extracts was incubated with agarose/anti-p21 conjugates for 4 hours at 4°C with rotation. Then, the agarose beads were washed 10 times with RIPA buffer (1X PBS, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM Na_3VO_4, 100 µg/ml PMSF, 20 µg/ml aprotinin, 10 µg/ml Leupeptin). Precipitated proteins were visualized by Western blot using appropriate antibodies (see figure legends for specifics).

Northern Blot for p21 mRNA. Total RNA was extracted by RNAqueous™ kit (Ambion, Houston, TX). Twenty µg of total RNA from each cell line was resolved on a 0.8% agarose gel containing formaldehyde and then blotted to nylon membranes by capillary transfer. The membranes were hybridized to a full-length p21 cDNA labeled with 32P-dCTP by Ready-To-Go™ DNA labeling kit (Amersham Pharmacia, Piscataway, NJ). The blot was hybridized overnight at 42°C with p21 probe in ULTRAhyb™ buffer (Ambion Inc, Houston, TX), washed three times for 5–10 minutes at room temperature with 2 x SSC and 0.1% SDS, and then three times for 15 minutes at 42°C with 0.1 x SSC and 0.1% SDS. The same membrane were stripped and rehybridized with β-actin probes. The hybridized p21 mRNA signals were scanned with STORM PhosphorImager and quantified by the SPOTFINDER softerware (Molecular Dynamics, Sunnyvale, CA).

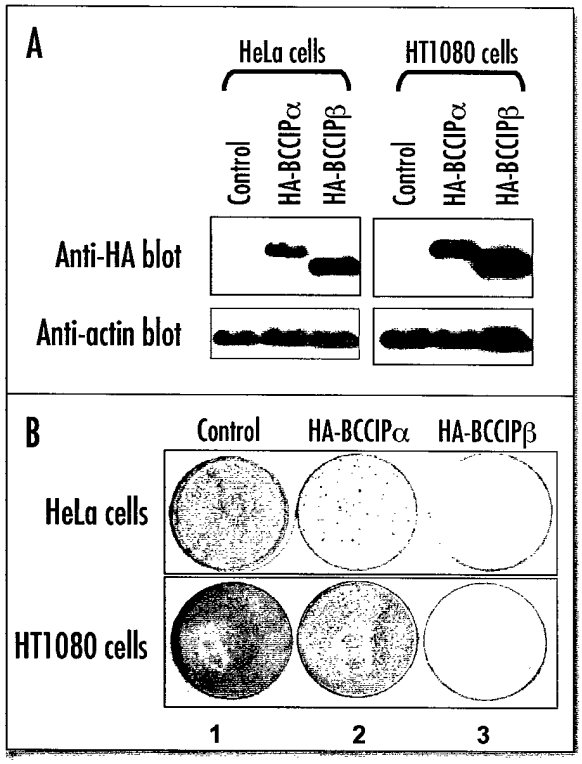

Figure 3. Expression of BCCIP reduces colony formation. (A) Expression of exogenous BCCIPα and BCCIPβ in HeLa and HT1080 cells shortly after the transfection. Half million of HeLa and HT1080 cells were transfected with 10 μg of plasmid vectors expressing HA-BCCIPα or HA-BCCIPβ. Control groups were transfected with vacant vector. Forty-eight hours after the transfection, equal amounts of protein were analyzed for HA-BCCIPα and HA-BCCIPβ expression by anti-HA-blot (top panel). Anti-actin blot (bottom panel) was used to confirm loading of equal amount of total proteins. (B) Inhibition of cell growth by BCCIPα and BCCIPβ identified by colony formation. Half million of HeLa and HT1080 cells were transfected with 10 μg of plasmid vectors expressing HA-BCCIPα (column 2) or HA-BCCIPβ (column 3). Control groups were transfected with vacant vector (column 1). Stably transfected cells were selected with puromycin and visualized by crystal violet staining after two weeks of selection.

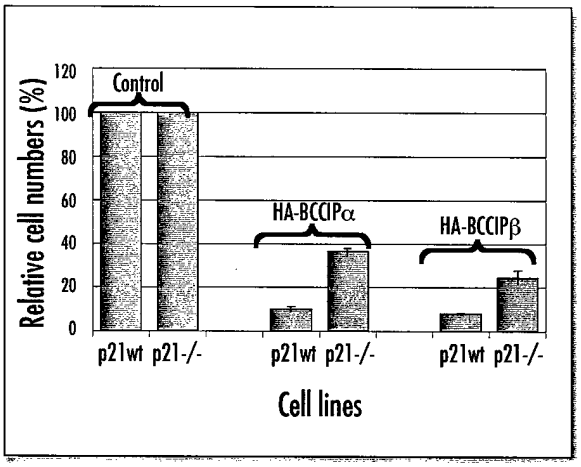

Figure 4. The inhibition of cell growth by BCCIP is partially dependent on p21 status. $p21^{-/-}$ and p21 wild type HCT116 cells, which are an isogenic set of human colon adenocarcinoma cell lines differing only in their p21 status, were seeded in 60mm plates at 1 x 10^6 cells/ml. Eighteen hours after the plating, the cells were transfected with equal amounts of plasmids that expressed HA-BCCIPα or HA-BCCIPβ and selected with puromycin for 2 weeks. Survival cells were then counted. Shown are the relative numbers of cells normalized to the same cell lines transfected with control vector. This figure shows that deficiency of p21 partially abrogates the cell growth inhibition conferred by HA-BCCIPα and HA-BCCIPβ.

RESULTS

Interactions Between Endogenous BCCIPβ and p21. The interaction between p21 and BCCIPα/Tok-1α was originally reported by Ono, et al.[2] Here, we reinvestigate the p21-BCCIP interaction for the following reasons. First, the interaction between BCCIPα and p21 was demonstrated by several methods, including yeast two-hybrid system, in vitro protein interaction assay using recombinant proteins purified from bacteria, coimmunoprecipitation with exogenously expressed p21 and BCCIP, and intracellular colocalization visualized by immunostaining. Yet, no confirmation using endogenous p21 and BCCIP proteins was documented.[2] Second, the p21-interaction domain was mapped to amino acids 161-259 of BCCIPα which are also present in BCCIPβ.[2] Although this implied a potential interaction between BCCIPβ and p21, the same report was not able to detect it using in vitro protein binding assays.[2] Third, BCCIPβ expression is more abundant than BCCIPα in many cell lines and tissues[1] and its expression was reduced in certain human tumor.[7] Therefore, it is imperatively important to clarify whether endogenous BCCIPβ interacts with p21. We covalently conjugated a monocolonal anti-p21 antibody to an agarose resin. This minimized the interference of soluble IgG in the subsequent immunoidentification of coprecipitated BCCIP proteins. As shown in Figure 1A, resin-conjugated with anti-p21 antibody effectively precipitated the endogenous BCCIPα and BCCIPβ, while nonspecific mouse IgG did not. To confirm this result, the whole cell extracts (WCE) from p21-deficient HCT116/$p21^{-/-}$ and the parental p21-proficient HCT116 cells were precipitated by the same anti-p21 agarose resin as in Figure 1A. As shown in Figure 1B, endogenous BCCIPα and BCCIPβ can only be precipitated from the p21 proficient cell, and not from the p21 deficient cells. These data confirmed that endogenous BCCIPα interacts with p21 and also suggested that BCCIPβ interacts with p21. However, BCCIPβ seems have a weaker interaction with p21 than BCCIPα.

Growth Inhibition by BCCIPβ. In a previous study, we reported that overexpression of BCCIPα moderately inhibits the growth of certain cancer cells.[1] These data were obtained from cell lines infected with retroviruses that constitutively overexpressed BCCIPα. Considering that BCCIPβ interacts with p21 and shares 258 identical N-terminal amino acids with BCCIPα, we tested whether expression of BCCIPβ may affect cell growth using the retroviral approach as described previously.[1] We found that retroviral-mediated overexpression of BCCIPβ indeed inhibited cell growth among the cell lines tested (Fig. 2). Furthermore, BCCIPβ inhibited cell growth at a greater extent than BCCIPα.

To confirm this result, colony formation experiments were performed. We transfected equal amounts of a BCCIPα and BCCIPβ expression vectors bearing a puromycin selection marker into HeLa and HT1080 cell and selected for puromycin-resistant cells for two weeks. Figure 3A shows the levels of exogenously expressed BCCIPα and BCCIPβ proteins shortly after the transfection. Transfection of vectors expressing BCCIPα or BCCIPβ resulted in fewer surviving colonies when compared to control (Fig. 3B). In addition, fewer surviving cells were observed on dishes transfected with BCCIPβ-expressing vector than that of BCCIPα (Fig. 3B). These data confirmed that BCCIPβ inhibits cell growth and suggested that BCCIPβ has a stronger inhibitory effect on cell growth than BCCIPα. The apparent discrepancy between this stronger inhibition on cell growth by BCCIPβ (Figs. 2 and 3) and the weaker BCCIPβ-p21 interaction (Fig. 1) will be addressed later.

The Inhibition of Cell Growth by BCCIP Can Be Partially Abrogated by p21 Deficiency. Because BCCIPα and BCCIPβ interact with p21, it is possible that BCCIPαβ may inhibit cell growth through a p21-dependent mechanism. To test this, we transfected equal amounts of BCCIPα and

BCCIPβ expression vectors into $p21^{-/-}$ and p21 wild type HCT116 cells, which are a pair of isogenic human colon adenocarcinoma cell lines differing only in their p21 status.[9] After selection of puromycin-resistant cells, we found that overexpression of either BCCIPα or BCCIPβ inhibits cell growth in p21 wild type HCT116 cells as expected (Fig. 4). How the inhibition of cell growth by BCCIPα or BCCIPβ is partially abrogated in $p21^{-/-}$ HCT116 cells. These data suggest that the growth inhibition of BCCIPαβ is at least partially dependent on a functional p21. However, the growth inhibition by BCCIP in $p21^{-/-}$ cells was not returned to the same level of the control, implying additional mechanism other than p21 may be responsible for further inhibition of cell growth. This remains to be identified.

Overexpression of BCCIPβ Causes Delayed G_1 to S Progression. To address the cellular mechanism by which BCCIPβ inhibits cell growth, we asked whether BCCIPβ inhibits the G_1/S transition as it interacts with p21. BCCIPβ and HT1080 cells were used because HT1080 has wild type p53, and BCCIPβ inhibited HT1080 growth more than BCCIPα did (Figs. 2 and 3). We constructed a Dox-inducible BCCIPβ overexpressing HT1080 cell line (Fig. 5A). As predicted, when BCCIPβ overexpression is induced, the growth rate of HT1080 cells is inhibited (Fig. 5B). To measure the G_1-S progression, we treated cells with 0.1 μg/ml nocodazole to block cells at M phase so that no new G_1 cells could be generated. Therefore, the gradual disappearance of the G_1 peak could be used to evaluate the dynamics of the G_1-S progression. As shown in Figure 5C, the G_1 phase peak in the control cells nearly disappeared by 8 hours after addition of nocodazole (columns 1 and 2), while a significant amount of BCCIPβ-overexpressing cells were retained in G_1 phase in the same period (column 3). Some BCCIPβ-overexpressing cells remained in G_1 phase for up to 24 hours after the nocodazole block was administrated (column 3). These data suggest BCCIPβ as an inhibitor for G_1-S progression, proving a cellular mechanism by which BCCIPβ inhibits cell growth.

Overexpression of BCCIPβ Results in Elevated p21 Levels. Although Figure 5 has provided cellular evidence for BCCIPβ as a regulator for G_1-S progression, the molecular mechanisms by which BCCIPβ regulates G_1-S progression is unclear. Ono et al suggested that the interaction between BCCIPα and p21 resulted in an enhanced inhibitory activity of p21 toward the histone H1 kinase activity of CDK2.[2] It would be logical to speculate that BCCIPβ also inhibits CDK2 activity because BCCIPβ interacts with p21. However, this does not explain a previously observed discrepancy between Figure 1 and Figure 2. Even though BCCIPβ had weaker interaction with p21 than BCCIPα (Fig. 1), it had a stronger inhibition on cell growth than BCCIPβα (Figs. 2 and 3). We sought for an alternative explanation.

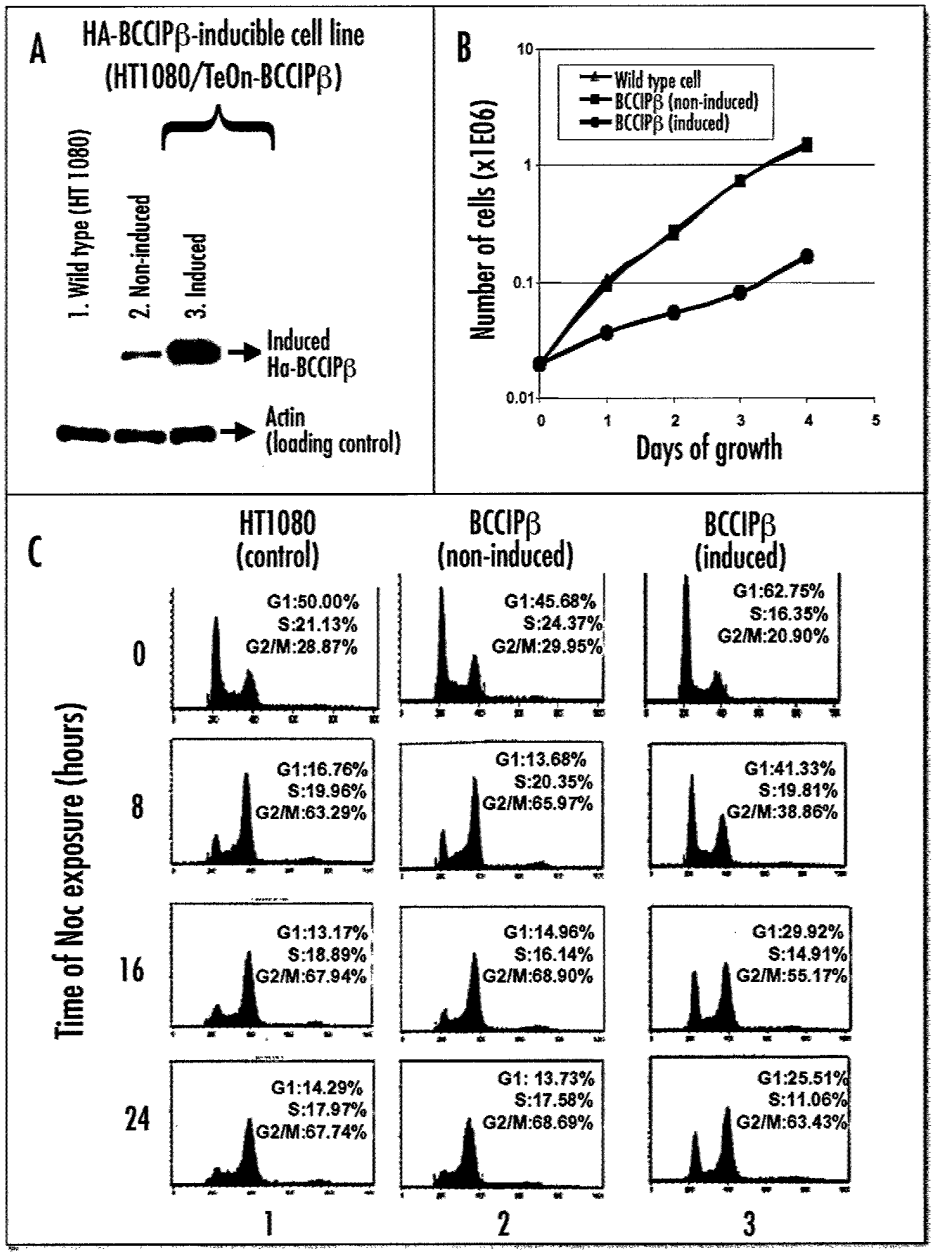

Figure 5. Induced BCCIPβ overexpression delays the G_1-S progression. (A) Doc-inducible BCCIPβ expression in HT1080 cells. HA-tagged BCCIPβ was cloned into pREV-TRE (Clontech), which was introduced via retroviral infections into HT1080 cells that had been previously transfected with pTet-On vector. This resulted in a Doc-inducible HA-BCCIPβ expressing cell line, designated HT1080/TetOn-BCCIPβ. Shown in the top panel is the exogenously expressed HA-BCCIPβ levels detected by anti-HA antibodies. The same membrane was reblotted with anti-actin (bottom panel) to confirm loading of equal amounts of protein for each sample. As shown, HA-BCCIPβ was significantly induced by addition of Doc in this cell line (lane 3) compared to noninduced condition (lane 2). The control cell line (lane 1) is the wild type HT1080 cells treated with Doc. (B) Induced BCCIPβ expression inhibits cell growth. Shown are the growth curves of a wild type HT1080 cells (triangle), HT1080/TetOn-BCCIPβ cells with noninduced BCCIPβ expression (square), and HT1080/TetOn-BCCIPβ cells with induced BCCIPβ expression (circle). This figure shows that induced BCCIPβ overexpression inhibits HT1080 growth. (C) Induced-BCCIPβ expression delays G_1-S progression. HT1080/TetOn-BCCIPβ cells as in panel A were treated with Doc for 3 days to induce BCCIPβ expression (column 3). Control groups are mock-induced HT1080/TetOn-BCCIPβ (column 2) or wild type HT1080 treated with Doc (column 1). Then, 0.1 μg/ml of nocodazole (Noc) was added to the cells. Various times after continuous Noc treatment (labeled on the left) cells were collected for flow cytometric DNA content analysis. The percentage of cells in G_1, S, and G_2/M are shown in each panel. The gradual disappearance of the G_1-peak represents the transition of G_1-phase cells into S-phase. This figure shows that overexpression of BCCIPβ delays the G_1-S transition.

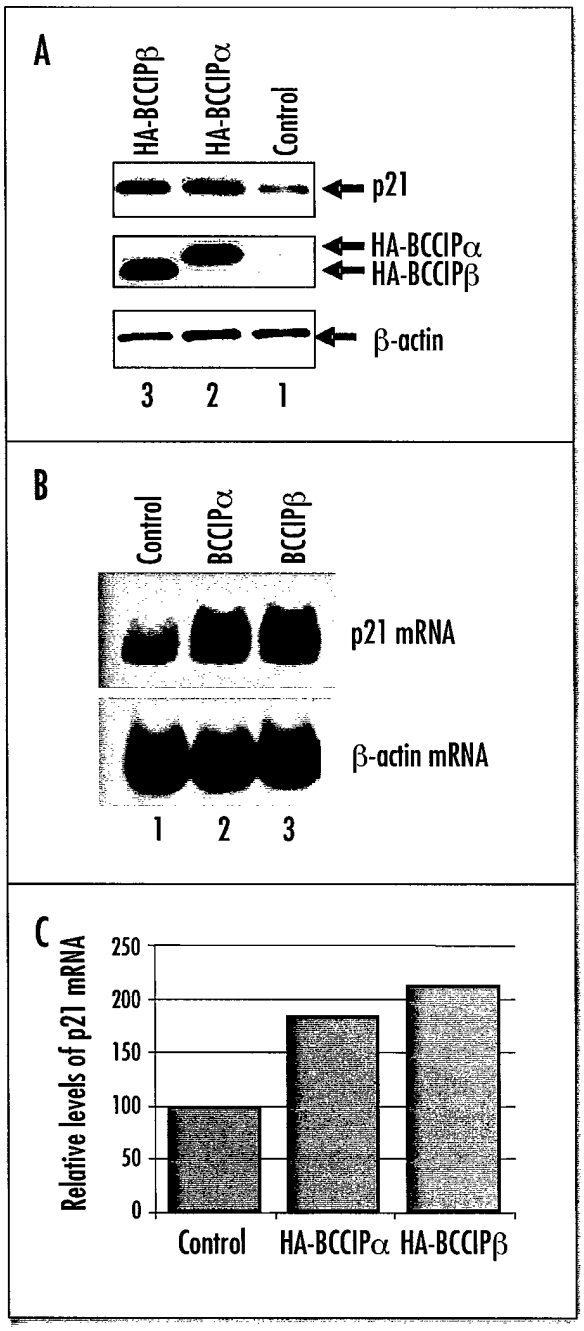

Figure 6. Overexpression of BCCIP stimulates p21 level. (A) Overexpression of BCCIP enhances p21 protein level. HT1080 cells were infected with HA-BCCIPα or HA-BCCIPβ expressing retroviruses and selected with puromycin. Then, p21 proteins in HT1080 cells overexpressing HA-BCCIPα (lane 2), HA-BCCIPβ (lane 3), or vacant vector (lane 1) were measured by anti-p21 blot (top panel). HA-antibody was used to detect the exogenously expressed HA-tagged BCCIPα or BCCIPβ (middle panel). Anti-β-actin antibody (bottom panel) was used to confirm loading of equal amount of total proteins in each lane. This figure shows that overexpression of BCCIPα and BCCIPβ enhanced p21 protein levels. (B) Northern blot of p21 mRNA in cells overexpressing BCCIP. Equal amounts of total RNA extracted from control (lane 1), HA-BCCIPα expressing (lane 2), and HA-BCCIPβ expressing (lane 3) cells were analyzed by Northern blots with p21 cDNA (top panel) and β-actin cDNA (bottom panel). This figure shows that expression of exogenous BCCIP results in elevated levels of p21 mRNA. (C) Quantification of relative p21 mRNA levels in HA-BCCIPα and HA-BCCIPβ expressing cells. Shown are quantified p21 levels as normalized to the actin mRNA level (see "Material and Methods" for details).

It was previously reported that BRCA2 protein inhibits the transcription activity of p53, and defect of BRCA2 results in elevated p53 and p21 protein levels.[10,11] Since BCCIP also interacts with BRCA2,[1] we hypothesized that overexpression of BCCIP may affect the p21 expression level as an alternative mechanism for BCCIP to regulate G_1-S transition. To test this, HA-tagged BCCIPα/β proteins were overexpressed in HT1080 cells, and the p21 protein and mRNA levels were measured. As shown in Figure 6, overexpression of BCCIP resulted in elevated p21 protein and mRNA levels. BCCIPβ increased p21 mRNA expression by 220%, while BCCIPα did so by ~180% (Fig. 6C). Furthermore, we did not observe a stabilization of p21 proteins in BCCIPα/β overexpressing cells after cycloheximide treatment (data not shown). This suggests an alternative mechanism by which BCCIP may regulate G_1 to S progression, i.e., stimulating p21 expression. In addition, BCCIPβ had a stronger effect on p21 mRNA expression than BCCIPα (Fig. 6C). This may explain why BCCIPβ inhibits cell growth more than BCCIPα does (Figs. 2 and 3) although it had a weaker interaction with p21 (Fig. 1).

DISCUSSION

Although both BCCIPα and BCCIPβ colocalize with p21 in nuclei, and the p21-binding domain is located in amino acids 161–258 of the common region between BCCIPα and BCCIPβ, a previous report did not identify an interaction between BCCIPβ and p21 in vitro.[2] The authors speculated that the BCCIPβ unique C-terminal region (amino acids 259 to 314) may have interfered with the binding between p21 and BCCIPβ. In this report, we demonstrate that both endogenous BCCIPα and BCCIPβ can actually coprecipitate endogenous p21 protein, although the interaction between p21 and BCCIPβ is relatively weaker than that of BCCIPα (Fig. 1). Therefore, our data clarified an important issue on BCCIP/p21 interaction.

Although the interaction between p21 and BCCIP suggests a role for BCCIP in G_1-S control, no cellular evidence has been documented before. Our data showed that BCCIPβ also inhibits cell growth and this inhibition is most likely due to an inhibition on the G_1-S progression during cell cycle. These data have provided cellular evidence that BCCIPβ is a new regulator for G_1-S progression. We further demonstrated that BCCIP regulate p21 expression as a mechanism by which BCCIP may regulate G_1-S progression.

The molecular mechanism by which BCCIP regulates G_1-S progression is largely unclear at this moment. However, several scenarios are worth discussion. First, the direct interaction between BCCIP and p21 may affect p21's effectiveness toward the CDK2 activity as suggested by One, et al.[2] Still, this mechanism cannot completely explain the phenotypes observed. Specifically, BCCIPβ showed a much weaker interaction with p21 than BCCIPα (Fig. 1), yet it has stronger inhibition on cell growth (Figs. 2 and 3). Second, overexpression of BCCIP may stimulate p21 expression as shown in Figure 6 via a previous unidentified mechanism. Obviously, the details of this mechanism remain to be elucidated. However, it is possible that overexpression of BCCIP may reduce BRCA2's inhibitory activity toward p53, since it was previously reported that BRCA2 inhibit p53's transcription activity;[10,11] BCCIP interacts with BRCA2,[1] and p21 expression is largely controlled by p53.[12-15] Third, it is known that the C-terminus of p21 is the substrate for AKT phosphorylation,[4,6,16] which regulates the PCNA-p21 interaction and the the redistribution of p21 protein between nucleus and cytoplasm.[6,16-18] Since BCCIP interacts with this C-terminal domain of p21, and BCCIP is largely a nuclear protein, it is possible that BCCIP may play a role in retaining p21 proteins in the nucleus thus promoting the inhibition of G_1-S progression. Alternatively, BCCIP may regulate

the G_1-S progression by modulating the p21-PCNA interaction, a scenario being investigated.

As shown in Figure 4, defect of p21 cannot completely abrogate the inhibition of cell growth by BCCIPαβ. It is possible that other CDK inhibitors, such as p27, may also be involved in the growth inhibition by BCCIP. It is intriguing that an elevated p27 protein expression can also be detected in cells overexpressing BCCIP proteins (Meng, et al. unpublished data). Therefore, it is possible that BCCIP may also inhibit cell growth and G_1-S progression via a p21-independent mechanism. In summary, our data suggest BCCIPβ as a new regulator for G-S progression in the cell cycle, which is partially related to its role in regulating p21 function.

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