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Rictor regulates FBXW7-dependent c-Myc and cyclin E degradation in colorectal cancer cells

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Abstract

Rictor (Rapamycin-insensitive companion of mTOR) forms a complex with mTOR and phosphorylates and activates Akt. Activation of Akt induces expression of c-Myc and cyclin E, which are overexpressed in colorectal cancer and play an important role in colorectal cancer cell proliferation. Here, we show that rictor associates with FBXW7 to form an E3 complex participating in the regulation of c-Myc and cyclin E degradation. The Rictor-FBXW7 complex is biochemically distinct from the previously reported mTORC2 and can be immunoprecipitated independently of mTORC2. Moreover, knocking down of rictor in serum-deprived colorectal cancer cells results in the decreased ubiquitination and increased protein levels of c-Myc and cyclin E while overexpression of rictor induces the degradation of c-Myc and cyclin E proteins. Genetic knockout of FBXW7 blunts the effects of rictor, suggesting that rictor regulation of c-Myc and cyclin E requires FBXW7. Our findings identify rictor as an important component of FBXW7 E3 ligase complex participating in the regulation of c-Myc and cyclin E protein ubiquitination and degradation. Importantly, our results suggest that elevated growth factor signaling may contribute to decrease rictor/FBXW7-mediated ubiquitination of c-Myc and cyclin E, thus leading to accumulation of cyclin E and c-Myc in colorectal cancer cells.

Keywords

rictor; FBXW7; c-Myc; cyclin E; colorectal cancer cells

Introduction

Mammalian target of rapamycin (mTOR) pathway, a critical integrator of growth factors in mammals, forms two distinct protein complexes, mTORC1 and mTORC2. mTORC1 is

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composed of mTOR, raptor, PRAS40 and mLST8/GβL, which is related to cell growth metabolism [1]. The second mTOR-containing complex, mTORC2, consists of mTOR, rictor, PROTOR/PRR5, sin1 and mLST8/GβL; less is known about the regulation and targets of mTORC2 [2]. By far, the best characterized function of mTORC2 is the phosphorylation of Akt at Ser473 [3]. Akt, a member of the AGC kinase family, influences cellular proliferation by inactivating cell cycle inhibitors (p27 and p21) and increasing cell cycle proteins (e.g., c-Myc and cyclin D1) [4; 5].

FBXW7, also called Cdc4 in yeast [6], is a member of the F-box family proteins, which interacts with Skp1 and Cdc53 to form a SCF-type ubiquitin ligase complex [7]. The hallmark of an F-box protein is a conserved domain of approximately 40 amino acids that mediates binding to Skp1 and defines the F-box. As an E3 component, FBXW7 regulates a network of substrates including c-Myc, cyclin E, c-Jun, Notch, mTOR and McI1 [8]. Most of FBXW7 substrates are important oncogenes that are overexpressed or activated in a wide range of human cancers. Accordingly, FBXW7 functions as a tumor suppressor; mutations of FBXW7 occur in diverse human tumor types [9]. Human FBXW7 encodes three transcripts, FBXW7α, FBXW7β and FBXW7γ; each isoform is localized in different subcellular compartments [10]. The FBXW7α isoform accounts for almost all of FBXW7 activity in the regulation of c-Myc and cyclin E [11].

Although rictor was originally identified as a novel binding partner of mTOR to regulate the cytoskeleton [12] and phosphorylate Akt, multiple complexes containing rictor have been identified and shown to be mTORC2 independent. For example, rictor interacts with integrin-linked kinase and regulates Akt phosphorylation and cancer cell survival [13]. Rictor-Myo1c complex participates in dynamic cortical actin events in 3T3-L1 adipocytes [14], and rictor interacts with PKCζ to regulate cancer cell metastasis [15]. Recently, rictor was shown to form a complex with cullin-1 to promote SGK1 ubiquitination and destruction; mTOR was not required for this interaction [16]. Phosphorylation of rictor at T1135 minimally affects the kinase activity of rictor in the mTORC2 complex but disrupts the interaction between rictor and cullin-1, leading to the reduction of SGK1 degradation [17]. These studies provide novel insights regarding unique functional roles of the rictor protein which are independent from its better characterized role as an interacting protein with mTOR. Here, we show that rictor forms a complex with FBXW7 and regulates the activity of FBXW7 leading to the ubiquitination and degradation of c-Myc and cyclin E.

Materials and Methods Materials

Materials

MG132 and cycloheximide (CHX) were purchased from Calbiochem (San Diego, CA). Rabbit monoclonal anti-rictor antibody, used for both immunoblotting and immunoprecipitation, was purchased from Bethyl Laboratories (Montgomery, TX). Rabbit anti-mTOR, rabbit anti-phospho-Akt (Ser473), mouse anti-Ubiquitin and rabbit anti-myc-tag antibodies were obtained from Cell Signaling (Beverly, MA). Mouse monoclonal anti-Flag-tag and anti-β-actin antibodies, and non-targeting control shRNA and rictor shRNA lentiviral particles were from Sigma (St. Louis, MO). Protein G PLUS-Agarose beads, rabbit anti-cyclin E and mouse anti-Akt1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-c-Myc antibody was from Epitomics (Burlingame, CA). The plasmids encoding c-Myc, myc-tagged rictor and myc-tagged mTOR were from Addgene (Cambridge, MA). The plasmid encoding Flag-tagged FBXW7α was kindly provided by Dr. Bruce E. Clurman (Fred Hutchinson Cancer Research Center, Seattle, WA). Human rictor and non-targeting control siRNA SMARTpools were purchased from Dharmacon (Lafayette, CO).

Cell culture and transfection

The human CRC cell lines SW620, HT29 and HCT116 were from ATCC (Manassas, VA). SW620 cells were maintained in DMEM supplemented with 10% of FBS. HT29 and HCT116 cells were cultured in McCoy's 5A supplemented with 10% of FBS. Wild-type and FBXW7^{-/-} HCT116 cells, kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD), were maintained in McCoy's 5A with 10% FBS. SW620 and HT29 cells were transfected with the siRNA duplexes and plasmids using electroporation (Gene Pulser; Bio-Rad, Hercules, CA) and lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively.

Stable rictor knockdown SW620, HT29 and HCT116 cells were generated. Cells were infected with the control shRNA or shRNA to human rictor lentivirus particles and stably expressing cells were selected with puromycin at a concentration of 2.5 µg/ml. The effective knockdown of rictor was monitored by western blot analysis.

Protein preparation and western blot analysis

Cells were collected at 48 h after transfection or at the indicated time points after treatment. Equal amounts of cell lysates were resolved on a 4–12% Bis-Tris gel and transferred to polyvinylidene fluoride membranes. Membranes were blocked by 5% non-fat milk for 1 h at room temperature. Rictor, mTOR, phospho-Akt (Ser473), Akt1, c-Myc, cyclin E, Myc-tag, Flag-tag and β-actin were detected with specific antibodies following blotting with a horseradish peroxidase-conjugated secondary antibody and visualized using a chemiluminescence detection system.

Immunoprecipitation and *in vivo* ubiquitination analysis

Cells were transfected with different plasmids as indicated, 1% Triton lysis buffer or CHAPS lysis buffer was used. After pre-incubation with protein G PLUS-Agarose beads, equal amount of protein (500 µg) were incubated with the indicated antibodies (1 µg) using an end-to-end rotor overnight at 4°C, followed by 4 h incubation with 25 µl of protein G PLUS-Agarose beads at 4°C. Indicated buffer was used to wash the beads three times. Reactions were stopped by adding 15 µl 2× loading buffer. Samples were denatured by boiling for 7 min and separated by NuPAGE 4–12% Bis-Tris gels. For *in vivo* ubiquitination analysis, his-ubiquitin plasmid was co-transfected with the indicated plasmids. Transfected cells were incubated with MG132 (20 µM) in serum free medium for 4 h before harvesting.

Results

Rictor regulates protein expression of c-Myc and cyclin E

Activation of Akt increases expression of c-Myc [18] and cyclin E [19]. Since inhibition of mTORC2 by knockdown of rictor inhibits Akt activation, we were interested to know whether rictor regulates c-Myc and cyclin E expression. Human colorectal cancer cells, SW620 and HT29, were transfected with shRNA targeting rictor and stable cell lines were established. Knockdown of rictor did not obviously affect the expression of c-Myc and cyclin E; however, with serum starvation, knockdown of rictor increased protein expression of c-Myc and cyclin E (Fig. 1A). In addition, treatment with MG132, a specific cell-permeable proteasome inhibitor, attenuated the increases of c-Myc and cyclin E protein expression resulting from rictor knockdown (Fig. 1B), indicating that the 26S-proteasome pathway was involved in this regulation. To further confirm our findings, we used rictor siRNA containing a different sequence from the rictor shRNA to decrease rictor expression. Consistently, knockdown of rictor by transient transfection with siRNA targeting rictor resulted in a significant increase of c-Myc and cyclin E protein expression (Fig. 1C). To further determine the role of rictor in the regulation of c-Myc and cyclin E protein

expression, SW620 and HT29 cells were transiently transfected with a myc-tagged rictor plasmid and the transfected cells were serum starved for 24 h before harvesting. As shown in Figure 1D, overexpression of rictor decreased c-Myc and cyclin E protein levels in both SW620 and HT29 cells, suggesting that rictor regulates the protein levels of c-Myc and cyclin E associated with serum deprivation. Considering the increases of c-Myc and cyclin E protein expression by Akt activation and knockdown of rictor resulting in the dephosphorylation and inhibition of Akt, our results demonstrate that rictor regulates c-Myc and cyclin E protein expression in an mTORC2/Akt pathway independent fashion.

Rictor interacts with FBXW7 to regulate c-Myc and cyclinE

FBXW7, an E3 component, targets c-Myc and cyclin E for degradation. Moreover, it has been shown that rictor forms a complex with cullin1 to degrade SGK1 protein [16]. To determine whether rictor participates in FBXW7-dependent regulation of c-Myc and cyclin E degradation, we generated rictor shRNA stable cell lines based on wild-type and FBXW7^{-/-} HCT116 cells. As shown in Figure 2A and 2B, knockdown of rictor induced the expression of c-Myc and cyclin E in wild type HCT116 cells but not in FBXW7^{-/-} cells. These data suggest that rictor regulation of c-Myc and cyclin E is FBXW7 dependent. We next determined whether rictor interacts with FBXW7. FBXW7 α is expressed at a much higher level than FBXW7 β and FBXW7 γ in most human cell lines, and usually plays a major role as an E3 ligase to the downstream targets [20]. Therefore, we transfected HCT116 cells with Flag-FBXW7 α together with myc-Rictor and the interaction between FBXW7 and rictor was detected. Two different lysis buffers were used in this assay: 1% triton and CHAPS lysis buffers. CHAPS was used as a mild buffer to keep mTORC2 intact while 1% triton was used to dissociate mTORC2 [21]. As shown in Figure 2C, Flag-FBXW7 α was clearly copurified when rictor was immunoprecipitated from cell lysates extracted with both 1% triton lysis buffer and CHAPS buffer, but the interaction between mTOR and rictor was only observed in the lysates extracted using CHAPS buffer. The interaction between rictor and the other two FBXW7 (β , γ) isoforms were also detected (data not shown). Although it has been reported that FBXW7 interacts with mTOR to promote its ubiquitination and degradation [22], our results indicate that rictor forms a complex with FBXW7 independent of mTORC2.

To further demonstrate the role of rictor in the FBXW7 E3 ligase complex, HCT116 control shRNA and rictor shRNA stable cell lines were transfected with empty vector (control) or Flag-FBXW7 α plasmid and c-Myc and cyclin E protein levels were analyzed (Fig. 2D). Overexpression of FBXW7 α decreased the protein levels of c-Myc and cyclin E (Fig. 2D) as expected. However, this degradation by FBXW7 α was significantly attenuated in rictor shRNA cells, demonstrating a role of rictor, bound with FBXW7 α as a part of E3 complex, to induce the degradation of c-Myc and cyclin E.

Rictor/FBXW7 promotes c-Myc and cyclin E ubiquitination

FBXW7 degrades c-Myc and cyclin E through the ubiquitination of c-Myc and cyclin E proteins. To address whether rictor promotes the ubiquitination of c-Myc and cyclin E, *in vivo* ubiquitination assays were performed. C-Myc or cyclin E and His-ubiquitin were expressed by transient transfection. Knockdown of rictor reduced the ubiquitination of both c-Myc (Fig. 3A) and cyclin E (Fig. 3C). As expected, the ubiquitination of c-Myc and cyclin E was diminished in FBXW7^{-/-} HCT116 cells compared with wild-type and, importantly, rictor-dependent regulation of c-Myc and cyclin E ubiquitination was also blunted in FBXW7^{-/-} HCT116 cells (Fig. 3B and 3D). Taken together, these data indicate a role of rictor in the regulation of c-Myc and cyclin E protein ubiquitination and degradation.

Rictor reduces the stability of c-Myc and cyclin E

To determine whether rictor regulates the stability of c-Myc and cyclin E, we next performed CHX chase assays. Time-course experiments showed that the half-lives of c-Myc (Fig. 4A) and cyclin E (Fig. 4C) were prolonged in HCT116 cells with rictor shRNA compared with HCT116 cells with non-targeting control shRNA. The sequence of rictor shRNA used in this study was designed to target the 3'-UTR region of rictor and would not affect the expression of transfected myc-rictor plasmid. Therefore, rictor protein levels can be rescued in rictor shRNA stable cell lines with myc-rictor overexpression. Indeed, transfection of HCT116 rictor shRNA stable cells with myc-rictor plasmid decreased the half-life of c-Myc (Fig. 4B) and cyclin E (Fig. 4D). Our results demonstrate that rictor is required for the integrity of the FBXW7 E3 complex to promote the degradation of c-Myc and cyclin E.

Discussion

Previously, the best characterized function of rictor associated with mTOR was the phosphorylation of Akt at Ser473. Activation of Akt promotes expression of c-Myc and cyclin E. In this study, we identified a distinct interaction between rictor and FBXW7 contributing to the degradation of c-Myc and cyclin E. Under serum deprived conditions, in which the mTORC2/Akt pathway is inhibited, rictor/FBXW7 functions as an E3 ligase complex, leading to the ubiquitination and degradation of c-Myc and cyclin E. Our results demonstrate a distinct regulation of c-Myc and cyclin E by the rictor/mTOR and rictor/FBXW7 complexes.

We have shown that rictor participates in the regulation of c-Myc and cyclin E only with serum deprivation. Recently, rictor was shown to form a complex with cullin-1 to promote SGK1 ubiquitination [16]; phosphorylation of rictor at Thr1135 by multiple AGC kinases including Akt, S6K and SGK, disrupts the interaction between rictor and cullin-1, resulting in the inactivation of rictor/cullin-1 E3 ligase complex [17]. In cells incubated in serum free medium, which resulted in low activity of AGC kinases, the majority of rictor was not phosphorylated at Thr1135, leading to the high activity of the rictor/cullin-1 complex. Conversely, in conditions associated with high AGC kinase activity, rictor was phosphorylated at Thr1135 by activated AGC, resulting in the dissociation of rictor from cullin-1 and inactivation of the rictor/cullin-1 E3 ligase complex. Our data show that rictor interacts with FBXW7 to form an E3 complex and regulates c-Myc and cyclin E protein degradation during serum starvation; however, the interaction between rictor and FBXW7 is not affected in cells cultured with or without serum (data not shown). These results suggest that the phosphorylation of rictor at Thr1135 may not affect the interaction between rictor and FBXW7. Further studies are required to determine whether phosphorylation of rictor at T1135 affects the activity of the rictor/FBXW7 complex targeting c-Myc and cyclin E.

Various growth factors such as insulin-like growth factor II and epidermal growth factor (EGF) and their receptors are overexpressed in colon cancer epithelial cells obtained from clinical samples [23]. Therefore, elucidating of the mechanisms of colon cancer cell proliferation in the presence of growth factors is important for a better understanding of colon carcinogenesis and in developing drugs against abnormal cell proliferation. Growth factors are known to promote tumor progression by upregulating important G1-S phase cell cycle proteins including c-Myc and cyclin E by activating phosphoinositide 3-kinase (PI3K)/AKT [23; 24; 25]. Our results indicate that c-Myc and cyclin E are subject to the ubiquitination by the Rictor/FBXW7 complex after serum deprivation, suggesting that elevated growth factor signaling, which is a hallmark of many types of cancers, contributes to decreased Rictor/FBXW7-mediated ubiquitination of c-Myc and cyclin E, thus leading to accumulation of cyclin E and c-Myc in colorectal cancer cells. c-Myc and cyclin E

overexpression has been reported in colorectal cancer [26; 27; 28]. Elevated growth factor signaling promotes tumor progression by upregulation of c-Myc and cyclin E not only through increased expression but also by reduced degradation. Thus, our work implicates a novel regulatory pathway for c-Myc and cyclin E, which may provide a novel mechanistic explanation for the frequent overexpression of c-Myc and cyclin E in colorectal cancers.

In conclusion, we show that rictor, a component of mTORC2, also forms an E3 ligase complex with FBXW7 to regulate the protein stability of c-Myc and cyclin E in colorectal cancer cells. Other complexes associated with rictor such as rictor/ILK, rictor/PKC ζ , rictor/Myo1c and rictor/cullin1 have also been reported [13; 14; 16]. Together, these novel findings add to our understanding of the multiple rictor complexes, independent of mTOR, which play a role in the regulation of cell proliferation.

HIGHLIGHTS

- Rictor associates with FBXW7 to form an E3 complex.
- Knockdown of rictor decreases ubiquitination of c-Myc and cyclin E.
- Knockdown of rictor increases protein levels of c-Myc and cyclin E.
- Overexpression of rictor induces the degradation of c-Myc and cyclin E proteins.
- Rictor regulation of c-Myc and cyclin E requires FBXW7.

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Figure 1. Rictor regulates protein expression of c-Myc and cyclin E

(A) SW620 and HT29 cells stably transfected with control shRNA or rictor shRNA were incubated in medium with or without 10% fetal bovine serum (FBS) for 24 h. Whole cell lysates were extracted and western blotting performed using the indicated antibodies. (B) SW620 and HT29 cells stably transfected with control shRNA or rictor shRNA were incubated in serum deprived conditions for 20 h followed by treatment with DMSO vehicle control or 20 μM MG132 for an additional 4 h. Whole cell lysates were used for western blot analysis. (C) SW620 and HT29 cells were transfected with control siRNA or siRNA targeting rictor. After a 24 h incubation in complete medium, cells were incubated in serum free medium for an additional 24 h. Transfected cells were lysed and western blot analysis

was performed using antibodies against rictor, c-Myc, cyclin E and β -actin. (D) SW620 and HT29 were transfected with control vector or a myc-rictor construct and incubated in complete medium for 24 h followed by incubation in serum-deprived conditions for an additional 24 h. Cells were lysed and western blot analysis was performed using antibodies against myc-tag, c-Myc, cyclin E and β -actin.

Figure 2. Rictor binds to FBXW7 and regulates c-Myc and cyclin E degradation

(A and B) Wild-type and FBXW7^{-/-} HCT116 cells were infected with lentiviral non-targeting control shRNA or lentiviral rictor shRNA construct and selected with puromycin. Cells were incubated in serum free medium for 24 h and western blot analysis was performed to detect c-Myc (A), cyclin E (B) and rictor (A&B) protein. (C) HCT116 cells were transfected with the indicated constructs. Forty-eight h post transfection, cells were lysed with the indicated lysis buffer and 1μg anti-rictor antibody was used to perform immunoprecipitation (IP) in each group. One μg normal IgG was used as a negative control. The anti-rictor immunoprecipitates were washed using the indicated lysis buffer and western blotting was performed using anti-flag, anti-mTOR and anti-rictor antibodies (left panel), respectively. Lysates (10μg) were used for input blotting (right panel). (D) HCT116 cells

containing non-targeting control shRNA or rictor shRNA were transfected with control vector or Flag-FBXW7a construct. Twenty-four h after transfection, cells were incubated in serum-deprived conditions for another 24 h, and whole lysates were isolated and analyzed by western blotting. C-Myc and cyclin E signals were quantitated densitometrically and expressed as fold-changes with respect to β -actin.

Figure 3. Rictor contributes to FBXW7-mediated c-Myc and cyclin E ubiquitination

(A and C) HCT116 cells containing non-targeting control shRNA or Rictor shRNA were transfected with His-ubiquitin and c-Myc (A) or cyclin E (C) plasmids. Forty-four post-transfection, cells were incubated in serum deprived conditions with DMSO vehicle control or 20μM MG132 for an additional 4 h. Immunoprecipitation analysis was performed using anti-c-Myc or anti-cyclin E antibody; normal IgG was used as a negative control. (B and D) Wild-type and FBXW7^{-/-} HCT116 cells, bearing non-targeting control shRNA or rictor shRNA, were transfected with His-ubiquitin and c-Myc (B) or cyclin E (D) plasmids. Forty-four h post-transfection, cells were incubated in serum-deprived conditions with DMSO vehicle control or 20μM MG132 for an additional 4 h. Immunoprecipitation analysis was

performed using anti-c-Myc or anti-cyclin E antibody; normal IgG was used as a negative control.

Figure 4. Rictor regulates the stability of c-Myc and cyclin E

(A and C) HCT116 control shRNA and rictor shRNA cells were cultured in serum free medium and treated with CHX (50 μg/ml) to block protein synthesis at different time points. Whole lysates were extracted and the protein levels of c-Myc (A) or cyclin E (C) were examined by western blot analysis. (B and D) HCT116 rictor shRNA cells were transfected with control (empty vector) or a myc-rictor plasmid. Twenty-four h after transfection, cells were incubated in serum-deprived conditions for another 24 h in the presence of CHX at the indicated time points. Whole lysates were used to perform immunoblotting. Quantification of the immunoblots is shown in the graphs below the blots.