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Association of Rictor with chromosomes during mitosis in MCF7 cells

O. M. Malanchuk

Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680
o.m.malanchuk@imbg.org.ua

The mTOR kinase forms two multicomponent protein complexes (mTORC1 and mTORC2), where protein Rictor is a unique component of mTORC2. These two complexes differ both structurally and functionally and transmit signals to distinct downstream substrates. In contrast to mTORC1, comparatively little is known about functioning and regulation of mTORC2 and its components. **Aim.** The analysis of the particularity of Rictor subcellular localization in MCF7 cells. **Methods.** Immunofluorescence assay using confocal microscopy was applied. **Results.** Analysis of Rictor localization with anti N-terminal mAb revealed a different pattern of immunostaining in cells that was depended on cell cycle phase. For the first time it was shown that Rictor is at the exposed edge of condensed chromatin pool in MCF7 cells. Furthermore Z-stacking of cells revealed that character of association depends on mitosis phases. **Conclusions.** Our novel findings add to our understanding of functioning of both Rictor and mTORC2 in cell cycle progression.

Keywords: Rictor, mTORC2, immunofluorescence, subcellular localization, mitosis.

Introduction

The mammalian target of rapamycin (mTOR) is kinase well conserved from yeast to mammals. It plays a central role in regulation of cellular homeostasis, cell growth and survival pathways by acting as a sensor for upstream inputs from multiple nutrient signals [1, 2]. In mammals, there are two multicomponent mTOR protein complexes [1, 2]: mTORC1 and mTORC2 which differs both structurally and functionally and signal to distinct downstream substrates.

The mTORC1 is composed of catalytic subunit mTOR, the regulatory-associated protein of mTOR (Raptor), mLST8 (also known as G β L), PRAS40, Deptor and the Tti1/Tel2 complex [3]. The activity of the mTORC1 complex is highly regulated in cells exposed to growth factors and nutrients and is sensitive to macrolide rapamycin [4]. The best-characterized mTORC1 kinase substrates include S6K and 4E-BP1 [4].

The mTORC2 complex includes catalytic subunit mTOR, rapamycin-insensitive companion of mTOR (Rictor), mSIN1, Protor-1/2, mLST8, Deptor and the Tti/Tel2 complex [5–10]. mTORC2 is relatively rapamycin-insensitive and thought to modulate growth factor signaling by phosphorylating the C-terminal hydrophobic motif of some AGC kinases such as Akt [11], SGK [12], and PKC alpha [13, 14]. In contrast to mTORC1, comparatively little is known about mTORC2 biology. It has been demonstrated that mTORC2 plays key roles in cell survival, metabolism, proliferation and cytoskeleton organization but the detailed mechanism remains to be addressed.

Although Rictor was originally identified as a novel binding partner of mTOR to regulate cell morphology and migration by playing a role as the indispensable component of mTORC2, it is likely that Rictor carrying mTORC2-independent functions also. In support of this notion Rictor has been found to associate with other proteins independently of mTOR, such as cul-

lin-1 (to promote SGK1 ubiquitination and destruction) [15], Myo1c (Rictor-Myo1c complex participates in dynamic cortical actin events in 3T3-L1 adipocytes) [16], integrin-linked kinase (ILK) (to regulate Akt phosphorylation and cancer cell survival) [17], and PKC ζ (to regulate cancer cell metastasis) [18].

To provide novel insights regarding functioning of mTORC2 complex as well as unique functional roles of the Rictor protein, which are independent from its better characterized role as an interacting protein with mTOR, subcellular distribution of Rictor during different stages of mitosis was analyzed. This analysis revealed for the first time that Rictor associates with chromosomes and this association depends on the stage of mitosis.

Materials and Methods

Cell culture and antibodies

MCF7 cell line was cultured at 37 °C in humidified incubator with 5 % CO₂. For routine cell culture, the human MCF7 were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Sigma, USA) supplemented with 10 % fetal bovine serum (FBS) (HyClone, Sigma, USA) and penicillin 50 unit/ml (Sigma, USA) and streptomycin 50 mkg/ml (Sigma, USA).

The mouse monoclonal antibody against Rictor was developed previously in our laboratory [19].

Immunofluorescence analysis

MCF-7 cells cultured on cover glasses were fixed with methanol for 5 min at room temperature. Thereafter, the cells were treated with 0.2 % Triton X100 in PBS for 15 min. The samples were incubated for 20 min in 10 mM cupric sulphate in 50 mM ammonium acetate, pH 5.0, to eliminate autofluorescence. Non-specific binding was blocked after incubation with 10 % FCS for 40 min at 37 °C. Antibodies obtained from clone D4 (anti-Rictor) were used in the concentration of 1 μ g/ml. The secondary FITC conjugated anti-mouse antibodies were applied in dilution 1:100 (Jackson ImmunoResearch, West Grove, PA, USA). As negative control MCF7 cells were incubated with secondary FITC conjugated anti-mouse anti-

bodies alone. Nuclei were counterstained with PI (Sigma, USA). All microscopy studies were performed using Zeiss LSM 510 META microscope (CarlZeiss Microscopy GmbH, Jena, Germany).

Results

Rictor distribution in interphase cells

Immunofluorescence assay using antibodies against Rictor protein previously generated in our laboratory [19] was performed on MCF-7 cell line. Mouse monoclonal antibody D4 mAb against N-terminal peptide of human Rictor is highly specific in Western blot and in immunofluorescence assay [19].

Here Rictor's subcellular distribution in human breast adenocarcinoma cell line MCF7 was examined. A uniform speckled cytoplasmic distribution of the endogenous Rictor as well as immunostaining of Rictor in nuclei was detected. Rictor is located on the nuclear envelope, and the nuclear pattern included punctate bodies, small dot-like speckles and speckles in all interphase cells (Fig. 1). Moreover, at the same sample changes in pattern of Rictor distribution in metaphase cells was detected: besides cytoplasmic Rictor distribution and its intense accumulation on condensed chromatin was observed. To shed a light on this phenomenon I focused on Rictor distribution at different stages of mitosis.

Distribution of Rictor in MCF-7 cells during mitosis

To determine the dynamic distribution of Rictor in the cells during mitosis, chromosomal DNA was stained with PI to show the cell phase (red), and Rictor was detected with (D4 mAb) and FITC conjugated goat anti-mouse IgG (see "Materials and Methods"). The samples were examined by confocal laser microscopy (Fig. 2). At prophase the chromatin begins to condense, the amount of Rictor small dot-like speckles and speckles were significantly increase comparing to interphase cells. At metaphase all chromosomes compacted on the equatorial plate and shaped as cylinder-like entity. The green fluorescent signals of the Rictor on borders of the cylinder were much stronger than that at the center.

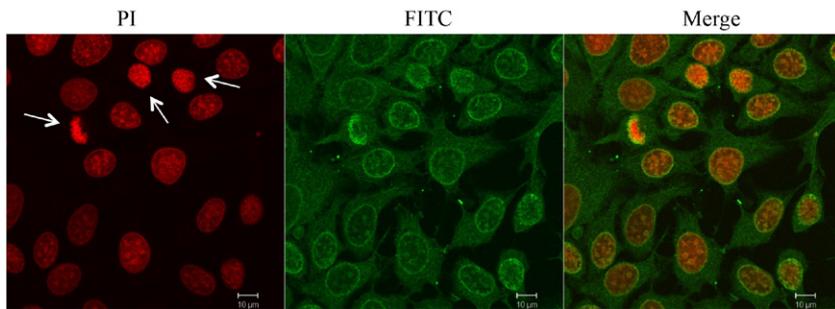


Fig. 1. Subcellular distribution of Rictor in MCF7 cells.

PI, chromosomal DNA stained with PI; **FITC**, Rictor stained by immunofluorescence with FITC conjugated antibody; **Merge**, merged image of the PI and FITC staining. Arrows indicate mitotic cells

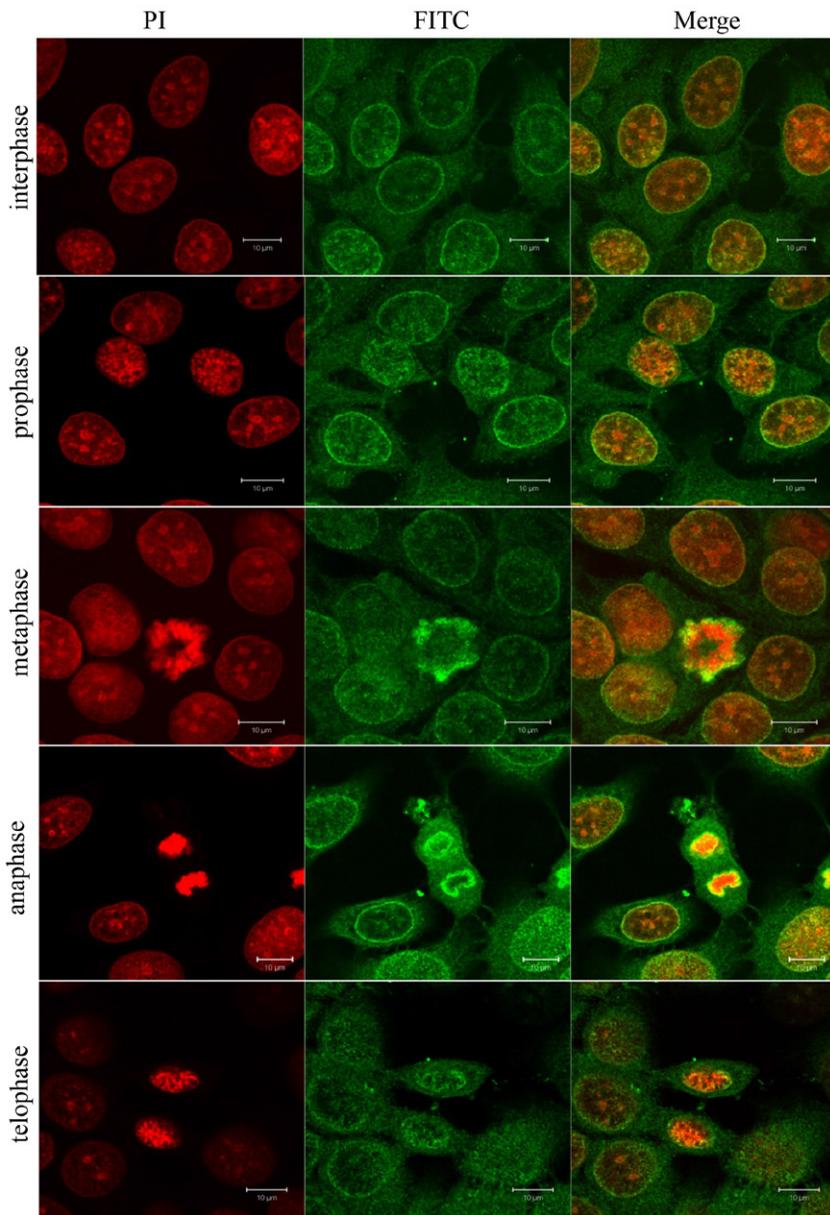


Fig. 2. Dynamic distribution of Rictor in interphase and at different stages of mitosis in MCF7 cells.

Column **PI**, chromosomal DNA stained with PI; Column **FITC**, Rictor stained by immunofluorescence FITC conjugated antibody; Column **Merge**, merged image of the PI and FITC staining

Some of strong fluorescent immunostaining was localization on the border of the cylinder of the condensed chromosome; namely, the Rictor protein location is overlapping with condensed chromosome (yellow). It indicates the association of Rictor with chromosomes. When sister chromosomes were getting apart after centromeric fission, the cells enter anaphase. There is stronger fluorescent signal of Rictor in the separating chromosomes in outer pool of condensed chromatin in compare to metaphase cells forming a “ring”-like struc-

ture. At telophase the separated chromosomes started to uncoil and became less condensed, immunofluorescence in outer pool of chromatin was considerably weaker and tended to one in interphase cells.

Determination of character of Rictor association with chromatin

To determine the character of Rictor association with chromatin Z-stacking was performed and allow us to obtain a composite image of multiple images taken

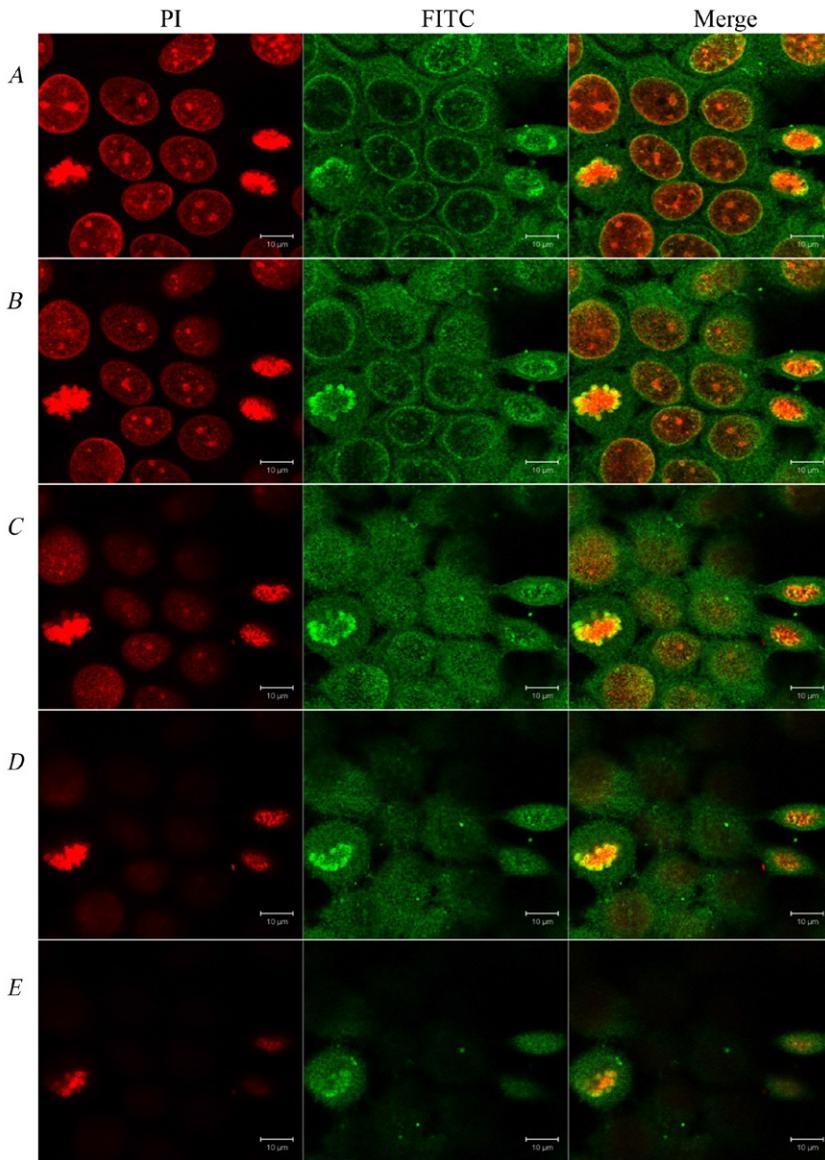


Fig. 3. Z-Stack of mitotic MCF7 cells. A – Z-stack was performed using confocal microscopy. A picture of the cell was taken on several planes, ranging from upper to lower levels. Images demonstrate that Rictor is exposed on the surface of condensed chromatin pool only (yellow). A. Upper image of cells. B – E. Every next image was taken in 1 μm deeper than previous one. Column PI, chromosomal DNA stained with PI; Column FITC, Rictor stained by immunofluorescence with FITC-conjugated antibody; Column Merge, merged image of the PI and FITC staining

at different focal distances [20]. 5 slices per Z-stack with 1 μm z-step of mitotic cells revealed that Rictor is localized at the exposed edge of condensed chromatin pool only (yellow) (see Fig. 3).

Discussion

There are controversial data about localization of mTORC1 and mTORC2 complexes and its components in the cell [21]. mTORC1 localization is well characterized in mammalian cells. Newest data demonstrate a wide distribution of mTORC1 in the plasma membrane, cytosol, lysosome and the nucleus although in some cases the evidence is questionable [21–23]. In contrast to mTORC1, the mTORC2 localization is more obscure. Recently Betz *et al.* revealed that mTORC2 localizes at mitochondria-associated ER membranes [24]. Another group shown that mTORC2 detected in a minor fraction on lipid rafts at the plasma membrane [18]. Moreover, there is evidence that mTORC2 is localized in the nucleus [24–27], while a nuclear function for mTORC2 is so far unknown. Intriguing, recently Sarbassov's group has discovered novel localization of mTOR on nuclear envelope where it associates with a critical regulator of nuclear import RanBP2 [28]. This association is dependent on the mTOR kinase activity and is not affected by inhibition of mTORC1 or deficiency of mTORC2 [28].

There are longstanding speculations about subcellular localization of Rictor and mTORC2. Although Rictor and mTORC2 can to be localized to the cytoplasm and the nucleus it is still unclear how translocation from the cytoplasm into the nucleus is regulated and what is the functional relevance of this transition.

In the present study, indirect immunofluorescent labeling and confocal laser microscopy was used to examine subcellular localization of Rictor protein and dynamic of its distribution in human breast adenocarcinoma cell line MCF7 at mitosis. The results show that Rictor has primarily uniform speckled cytoplasmic distribution. Immunostaining of Rictor in the nucleus indicates its location on the nuclear envelope, and the varying minor nuclear staining pattern in interphase cells. The detailed analysis of mitotic MCF7 cells revealed the different pattern of Rictor distribution characterized by accumulation

on condensed chromatin and gradual increasing of its association through mitotic stages. Moreover the dynamic distribution of Rictor in the MCF7 cells is correlated with mitosis progress temporally and spatially: Rictor was at the exposed edge of condensed chromatin pool only; the weak Rictor association with condensed chromatin started in minor at prophase of MCF7 cells and reached maximum at anaphase.

Chromatin is the complex of DNA and proteins that are packed within the nucleus of mammalian cells. Up to date little is known about the interaction of Rictor with DNA or DNA-binding proteins, such as histones or non-histone proteins. Rictor possesses two domains in its N-terminus (ARM-like and ARM-type fold), which are the super-helical structures and presents an extensive solvent-accessible surfaces that are well suited to binding large substrates such as proteins and nucleic acids [29]. So, hypothetically, there is sufficient prerequisite for direct Rictor interaction with chromosomal DNA. The biological function of such interaction remains to be elucidated.

The other possibility is that Rictor binds to histones and thus determines the target of phosphorylation by mTORC2 whereupon phosphorylated histone gets involved in cytokinesis. But it is unclear whether such late histone phosphorylation is possible.

However, strong Rictor spreading all over the chromosomes at metaphase and anaphase may suggest that it is able to bind exposed the telomeric regions of chromosomes. This binding might be either direct or indirect, mediated by telomere binding proteins and play role in maintaining of chromosome stability.

The detection of one component of TORC2 complex does not necessarily reflects localization of an entire complex. However, it is known that a majority of the Rictor staining overlaps with mTOR and this observation makes Rictor a valuable marker in pursuing the subcellular localization of mTORC2 [30]. In conclusion, our findings are in agreement with the notion that mTOR complexes are hyperactive during mitosis and suggest that all or at least part of their components (e.g., Raptor [31–34] and Rictor) may physically and functionally interact with the mitotic and cytokinetic apparatus during cell division. It re-

mains to be demonstrated the functional significance of Rictor association with chromatin as well as to determine its interacting target (DNA or proteins), besides to be elucidated whether Rictor acts alone or in complex with mTOR, and finally the precise mechanism of regulation of Rictor association during mitosis and cytokinesis.

Altogether, these novel findings add to our understanding of the Rictor complex, independent of mTOR, which may play a role in the regulation of cell proliferation. It will be of great interest for the future to investigate the molecular mechanisms by which mTORC2 complex or Rictor alone are functioning and are regulated in normal and malignant cells, which will advance our understanding of this signaling network and facilitate the identification of potential targets for treating human cell division-related diseases, including cancer and aging.

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Асоціація Rictor з хромосомами під час мітозу в клітинах лінії MCF7

Маланчук О. М.

Кіназа mTOR формує два мультикомпонентних комплекси (mTORC1 та mTORC2, унікальним компонентом останнього є Rictor), які відрізняються як структурно, так і функціонально, та мають різні мішені. На відміну від mTORC1, відносно мало відомо про функціонування і регуляцію комплексу mTORC2 і його компонентів. **Мета.** Аналіз особливостей субклітинної локалізації Rictor в клітинах MCF7. **Методи.** Імунофлуорисцентний аналіз із використанням конфокальної мікроскопії. **Результати.** Аналіз локалізації Rictor за допомогою моноклональних антитіл до N-термінального фрагменту Rictor продемонстрував диференційний патерн імунофарбування клітин в залежності від стадії клітинного циклу. Вперше виявлено значну поверхневу асоціацію Rictor з конденсованим хроматином в клітинах MCF7. Більш того, Z-стекинг клітин дав можливість зрозуміти характер асоціації, яка, як виявилось, залежить від фази мітозу. **Висновки.** Дані, отримані нами вперше, вносять вклад в розуміння функціонування як білка Rictor, так і комплексу mTORC2 в прогресії клітинного циклу.

Ключові слова: Rictor, mTORC2, імунофлуорисценція, субклітинна локалізація, мітоз.

Асоціація Rictor с хромосомами во время митоза в клетках линии MCF7

Маланчук О. Н.

Киназа mTOR формирует два мультикомпонентных комплекса (mTORC1 и mTORC2, уникальным компонентом последнего является Rictor), которые отличаются как структурно, так и функционально и имеют разные мишени. В отличие от mTORC1, относительно мало известно о функционировании и регуляции комплекса mTORC2 и его компонентов. **Цель.** Анализ особенностей субклеточной локализации Rictor в клетках MCF7. **Методы.** Иммунофлуорисцентный анализ с использованием конфокальной микроскопии. **Результаты.** Анализ локализации Rictor с помощью моноклональных антител к N-терминальному фрагменту Rictor выявил дифференциальный паттерн иммуноокрашивания клеток в зависимости от стадии клеточного цикла. Впервые удалось показать значительную поверхностную ассоциацию Rictor с конденсированным хроматином в клетках MCF7. Более того, Z-стекинг клеток позволил понять характер ассоциации, которая, как оказалось, зависит от фазы митоза. **Выводы.** Данные, полученные нами впервые, вносят вклад в понимание функционирования как белка Rictor, так и комплекса mTORC2 в прогрессии клеточного цикла.

Ключевые слова: Rictor, mTORC2, иммунофлуорисценция, субклеточная локализация, митоз.

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