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Endoplasmic reticulum is a main localization site of mTORC2

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ABSTRACT

The Akt kinase is a critical effector in growth factor signaling. Activation of Akt driven by the growth factor dependent PI3K (phosphatidylinositol-3-OH kinase) is coupled to the plasma membrane translocation and phosphorylation of Akt on two sites by PDK1 (phosphoinositide-dependent protein kinase-1) on Thr-308 and by mTORC2 (mammalian Target of Rapamycin Complex 2) on Ser-473. In our study we examined the sub-cellular localization of mTORC2 and identified that this kinase complex predominantly resides on endoplasmic reticulum (ER). Our immunostaining analysis did not show a substantial co-localization of the mTORC2 component rictor with Golgi, lysosome, clathrin-coated vesicles, early endosomes, or plasma membrane but indicated a strong co-localization of rictor with ribosomal protein S6 and ER marker. Our biochemical study also identified the mTORC2 components rictor, SIN1, and mTOR as the highly abundant proteins in the ER fraction, whereas only small amount of these proteins are detected in the plasma membrane and cytosolic fractions. We found that growth factor signaling does not alter the ER localization of mTORC2 and also does not induce its translocation to the plasma membrane. Based on our study we suggest that the mTORC2-dependent phosphorylation of Akt on Ser-473 takes place on the surface of ER.

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1. Introduction

Growth factor peptides regulate cell growth, proliferation, survival, and metabolism by activation of the phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway [1]. PI3K nucleates growth factor signaling on the plasma membrane by functioning as a kinase of phosphatidylinositol-4,5-diphosphates and generating phosphatidylinositol-3,4,5-triphosphates (PIP3s). The plekstrin homology (PH) domain of Akt binds to PIP3s and recruits Akt to plasma membrane. This translocation step is critical in regulation of Akt because at the plasma membrane its phosphorylated on Thr-308 and Ser-473 sites required for its full activation [2]. The second Ser-473 site known as the regulatory hydrophobic motif site of Akt is phosphorylated by the kinase complex known as mTORC2 (mammalian Target of Rapamycin Complex 2) [3].

Mammalian target of rapamycin (mTOR) is a central component of the essential and highly conserved signaling pathway that controls anabolic processes in cells [3]. The biochemical studies revealed that yeast TOR [4] or mammalian TOR (mTOR) with its interacting proteins mLST8 and DEPTOR exists at least in two distinct complexes [5]. Its first complex mTORC1 (mTOR Complex 1) assembled by mTOR and its binding proteins raptor. It functions as a nutrient-sensing complex and controls cell growth and cell size. The second complex mTORC2 is assembled by mTOR and its interacting proteins rictor and SIN1. The functional studies defined mTORC2 as the regulatory kinase of the distinct members of AGC (protein kinase A, G, and C) family including Akt, PKCa, and SGK1 [5].mTORC2 regulates Akt by phosphorylation of its two different sites. The mTORC2-dependent phosphorylation of Akt on the regulatory Ser-473 site is dependent on growth factor signaling, whereas a basal activity of mTORC2 maintains the constitutive phosphorylation of Akt on its turn motif Thr-450 site [2]. This difference indicates that phosphorylation of the Thr-450 and Ser-473 sites on Akt by mTORC2 are separate events and might take place at different locations. It has been proposed that translocation of Akt to the plasma membrane coupled with its phosphorylation on Thr-308 and Ser-473 is a critical step in activation of Akt by growth factor signaling [2]. The functional localization of mTORC2 has been supported by the recent finding that in yeast TORC2 has been identified at the plasma membrane [6]. It has been also reported that mTORC2 in association with ribosomes promotes cotranslational phosphorylation of Akt on Thr-450 [7]. Importantly, activation of mTORC2 by association with ribosome has been demonstrated by Hall group [8]. Currently, the functional localization of mTORC2 remains elusive. In our present study we have addressed this problem by examining the sub-cellular localization of mTORC2.

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2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: DMEM/F12 from Life Technologies; the Fetal Bovine Serum (FBS) from Hyclone, Fugene 6 transfection reagent and the complete protease inhibitor cocktail from Roche; for immunoblotting we used the following antibodies: rictor, HRP-labeled anti-rabbit/anti-mouse/anti-goat secondary antibodies, and tubulin from Santa Cruz Biotechnology and mTOR from Cell Signaling. The Alexa-coupled anti-mouse/anti-rabbit secondary antibodies from Invitrogen. For immunostaining we used the following set of antibodies: rictor (Santa Cruz #sc-50678), mTOR (Strategic Diagnostics Inc. #28130002, presently distributed by Novus Biologicals by the same catalog number), LAPM2 (BD Bioscience #555803) EEA1 (BD Bioscience #610456), Clathrin (Cell Signaling #2410), GM130 (Cell Signaling #2296), Calreticulin (Abcam #ab22683).

2.2. Cell lines and culture

MDA-MB-435 and A549 were obtained from American Type Culture Collection. MDA-MB-435 and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% FBS. The cells reached 70–80% confluency were serum starved for 24 h and stimulated by incubation with insulin-like growth factor I (IGFI) 50 ng/ml for 20 min.

2.3. Lentivirus production, and infection

Lentiviral shRNAs targeting human mTOR and rictor were generated and used as described previously [9]. Lentiviruses were harvested 48 h after transfection and spinned at 3000*g* for 15 min in order to eliminate any remaining 293T cells. The viral supernatant was added at a ratio of 1:1 to the culture medium in the presence of polybrene (8 μg/ml) and the cells were spinned at 1200*g* for 45 min at 32 °C, in order to increase the infection efficiency. Cells incubated with the retroviruses for the following 24 h. After an additional 24 h of recovery in normal medium, infected cells were passaged and selected with puromycin (2.5 mg/ml for 3–4 days). To express ER-GFP the cells were infected by the Organelle Lights™ ER-GFP virus (Invitrogen #O36212).

2.4. Cell lysis and immunoblotting

Cells were rinsed with ice-cold PBS before lysis in buffer containing as described previously [9]. The scraped lysates were incubated for 20 min at 4 °C to complete lysis. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm (17,500*g*) for 10 min in a microcentrifuge. The protein concentration was measured using the 5× Biorad protein assay and the samples were denaturated in Laemmli buffer. 10 μg of protein were resolved by SDS–PAGE (7.5% acrylamide) and transferred to PVDF membrane. Proteins were analyzed by immunoblotting and detected by enhanced chemoluminescence (ECL).

2.5. Immunofluorescence

For endoplasmic reticulum (ER) staining, cells were pre-treated, two days before the experiment, with the Organelle Lights™ Reagents (Invitrogen) according to the manufacturer's instruction. This technique is based on expression, via baculovirus delivery system, of fluorescent protein-signal peptide fusions for accurate and specific targeting to subcellular compartments and structures. Chamber slide were coated with fibronectin 10 μg/ml in PBS (3 $\mu g/cm^2$) and incubated at 37 °C. After 2 h, extra fibronectin was removed and cells were plated: 30,000 cells per dish for A549 and 100,000 cells per dish for MDA-MB-435. Twenty-four hours after plating, cultured cells were fixed with 3.7% paraformaldehyde for 15 min. After three washes with PBS, cells were permeabilized with 0.1% Saponin in PBS for 20 min. After another three PBS washes, free aldehydes were blocked with 50 mM ammonium chloride for 20 min followed by blocking of non specific sites with 10% horse serum diluted in PBS for 1 h. The cells were then incubated with the appropriate primary antibodies overnight at 4 °C in a humid chamber. The labeled proteins were detected using the appropriate Alexa 488and/or Alexa 594-conjugated secondary antibodies for 1 h at room temperature. The cells were then incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min. After three last PBS washes, the slides were quickly rinsed into water and mounted in Mowiol based mounting medium. Finally, slides were observed and pictures were taken using Olympus IX71FV500 Laser Scanning Confocal Microscope with UV excitation laser. The resulting images were analyzed using the software Fluoview 5.0, according to the manufacturer's instructions (http://www.olympusfluoview.com/java/colocalization/index.html).

2.6. The biochemical sub-cellular fractionation

The cytosolic and endoplasmic reticulum (ER) fractions have been purified as described previously [10]. Briefly, we utilized the discontinuous sucrose gradients to purify ER. Initially, cells are lysed mechanically with sonication and, then, a low-speed centrifugation (700*g*) is used to remove large cellular debris. Supernatant from this step is collected as a total lysed protein fraction. A subsequent 15,000*g* centrifugation crudely pellets mitochondria and separates it from ER and other organelles. The supernatant is loaded onto a three-layered sucrose gradient and purified ER is banded by centrifugation at 152,000*g*. ER band accumulates at the interface of 1.3 M sucrose. The plasma membrane fraction was isolated by using BioVision kit (#K268-50) as recommended by the manufacture.

3. Results

3.1. Detection of mTORC2 by the co-localization of rictor and mTOR staining

Rictor is an essential component of mTORC2 [3]. To initiate our sub-cellular localization study of mTORC2, we pursued the rictor localization by immunostaining. First, we optimized the immunostaining of rictor by screening a set of the rictor antibodies and cellular fixation/permeabilization conditions in MDA-MB-435 cells. The permeabilization of cells following the fixation step with a low concentration of saponin has been selected as the most effective in detection of rictor. A specificity of the rictor detection in this condition has been validated by the knock down of rictor by the lentiviral expression of its targeting shRNA. The efficiency of the rictor knock down and deficiency of the mTORC2 signaling has been examined by immunoblotting (Fig. S1 A and B). Following the knock down of rictor expression as indicated by the immunoblotting of cellular lysates, in the absence of rictor we detected only a low background signal by immunostaining. The rictor staining shows a perinuclear and cytoplasmic dotted pattern implying that rictor is co-localized with cellular organelles. In our next step by a similar approach we have validated a specificity of the mTOR immunostaining (Fig. S2 A and B). We observed a similar dotted pattern of the mTOR staining only in control but not in the mTOR knock down cells indicating a specific detection of mTOR by immunostaining.

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