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mTOR/S6K1 and MAPK/RSK signaling pathways coordinately regulate estrogen receptor α serine 167 phosphorylation

Rachel L. Yamnik [a], Marina K. Holz [a,b,*]

[a] Department of Biology, Stern College for Women of Yeshiva University, New York, NY 10016, USA
[b] Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

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ABSTRACT

Resistance to anti-estrogen therapy is a major clinical concern in treatment of breast cancer. Estrogen-independent phosphorylation of estrogen receptor α, specifically on Ser167, is one of the contributing causes to development of resistance, and a prognostic marker for the disease. Here, we dissect the signaling pathways responsible for Ser167 phosphorylation. We report that the mTOR/S6K1 and MAPK/RSK contribute non-overlapping inputs into ERα activation via Ser167 phosphorylation. This cooperation may be targeted in breast cancer treatment by a combination of mTOR and MAPK inhibitors.

1. Introduction

The mammalian target of rapamycin (mTOR) is a conserved protein kinase that is a key regulator of cell growth and proliferation in response to nutrient availability and growth stimuli. Rapamycin is a naturally-derived inhibitor of mTOR, and an inhibitor of cell proliferation, as manifested by its potent immunosuppressive properties and activity against solid tumors [1]. Recent work led to the realization that rapamycin does not perturb all mTOR functions because mTOR exists in two complexes in eukaryotic cells, mTOR complexes 1 and 2 (mTORC1 and 2). mTORC1 and mTORC2 consist of distinct sets of proteins and perform non-redundant functions [2]. This work focuses on the rapamycin-sensitive mTORC1 signaling.

In response to a variety of stimuli, including mitogens and hormones, the mitogen-activated protein kinase (MAPK) and mTORC1 pathways regulate important cellular processes such as cell growth, proliferation, and survival [3,4]. There exists an extensive cross-talk between MAPK and mTORC1 signaling in cells. Correspondingly, the effectors of these pathways, the p90 ribosomal S6 kinase (RSK) and the p70 S6 kinase 1 (S6K1) have been shown to converge on a common set of targets, most notably in control of protein translation [5–7]. In this study, we identify estrogen receptor α (ERα) as a recipient of coordinated phosphorylation inputs from the MAPK and mTORC1 pathways.

ERα mediates the proliferative effects of estrogen and represents an important clinical target in treatment of breast cancer. Tamoxifen is an anti-estrogen that has become the standard agent for the treatment of ER-positive breast cancer, where it acts as an antagonist. However, resistance to tamoxifen, and other endocrine or anti-estrogen therapies develops in many cases [8,9]. One mechanism by which resistance develops is through phosphorylation of ERα, allowing it to act in estrogen-independent manner. As illustrated in Fig. 1, the N-terminal estrogen-independent activation AF-1 domain of ERα is responsible for ligand-independent transactivation function of ERα. ERα phosphorylation within the AF-1 domain occurs on residues Ser104/106, Ser118, and Ser167. Ser104/106 phosphorylation is regulated by cdk [10], and Ser118 phosphorylation is regulated by MAPK [11,12], although it has been suggested that MAPK controls this event indirectly [13]. Phosphorylation of Ser167 has been previously attributed to Akt and RSK [14,15], while we have demonstrated that S6K1 is the physiological ERα Ser167 kinase and it phosphorylates this site in

Abbreviations: S6K1, p70 S6 kinase 1; MAPK, mitogen-activated protein kinase; RSK, p90 ribosomal S6 kinase; mTOR, mammalian target of rapamycin; ERα, estrogen receptor α; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C

* Corresponding author. Address: Department of Biology, Stern College for Women of Yeshiva University, New York, NY 10016, USA. Fax: +1 212 340 7883.
E-mail address: mholz@yu.edu (M.K. Holz).

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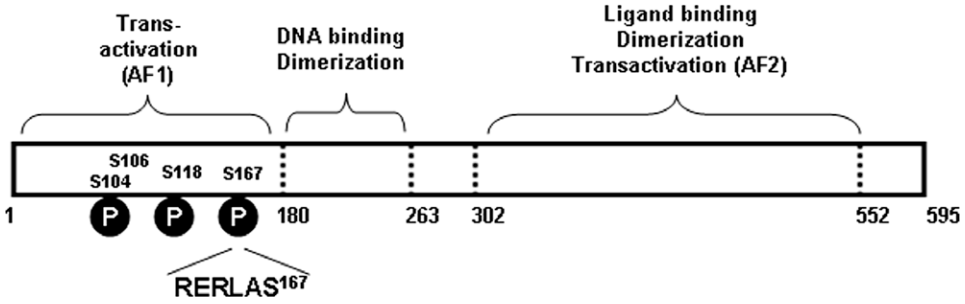

Fig. 1. Domain architecture of estrogen receptor α (ERα), and location of phosphorylation sites within the AF-1 domain.

rapamycin-sensitive fashion [16]. Importantly, Ser167 phosphorylation correlates with resistance to tamoxifen [14] and is a prognostic marker for disease progression and survival [17]. Thus, the identity of the kinase(s) responsible for this phosphorylation event has important clinical consequences.

RSK and S6K1 recognize identical consensus phosphorylation sequence RxRxxS/T, where x is any amino acid, and they share common phosphorylation targets [5,6]. ERα contains a phosphorylation motif RERLAS^{167} (Fig. 1), and both kinases have been shown to directly phosphorylate this site in in vitro kinase assays [15,16]. Because of the different kinetics of mitogen-mediated activation of the mTORC1/S6K1 and MAPK/RSK signaling pathways, it is possible that RSK may play a physiological role in phosphorylation of ERα. Therefore, we set out to determine the relative contributions of the MAPK/RSK and mTORC1/S6K1 signaling pathways to phosphorylation and activation of ERα. In this study, we demonstrate that in response to activating stimuli S6K1 and RSK phosphorylate ERα, allowing for coordinate regulation of ERα activation.

2. Materials and methods

2.1. Reporter and expression vectors

pGL2-3xERE-TATA-luc was kindly provided by Donald P. McDonnell (Duke University, Durham, NC), and pIS2 renilla luciferase reporter was kindly provided by David Bartel (MIT, Cambridge, MA).

2.2. Cell culture

MCF7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

2.3. RNAi against RSK1/2

For the siRNA studies, double-stranded RNAs for RSK1 and RSK2 were a kind gift from John Blenis (Harvard Medical School, Boston, MA). MCF7 cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's recommendations. After 24 h post-transfection, cells were deprived of serum overnight, treated with agents as indicated in the figure legend.

2.4. Reporter gene assays

For luciferase reporter assays, cells were transfected using Lipofectamine2000 (Invitrogen) using the manufacturer's protocol with plasmids encoding for firefly luciferase under control of three ERE, and control renilla luciferase. At 24 h post-transfection, rapamycin (20 ng/mL) and/or U0126 (Biomol, 10 μM) were added where indicated. At 48 h post-transfection, cells were harvested using 1× Passive Lysis Buffer (Promega), and relative luciferase activity was measured using the Dual Luciferase Reporter Assay System and Glomax 20/20 luminometer (Promega).

2.5. Immunoblots

Cells were lysed using 1× Passive Lysis Buffer (Promega). Whole-cell lysates (10% of total cell extract) were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (10%). Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) and blotted with the indicated antibodies. Anti-phospho-ERK1/2 antibodies were from Sigma. Anti-phospho-S6K1 Thr389, anti-phospho-S6 Ser235/236, anti-phospho-S6 Ser240/244, and anti-phospho-ER Ser167 antibodies were purchased from Cell Signaling Technology. Anti-ER antibodies were from Santa Cruz Biotechnology. Anti RSK1 and RSK2 antibodies were from Zymed. For immunoblotting, anti-rabbit, anti-mouse, and anti-goat horseradish peroxidase (HRP)-conjugated antibodies were purchased from Amersham, Chemicon, and Santa Cruz Biotechnology, respectively. Immunoblots were developed using enhanced chemiluminescence reagents (Pierce) and Chemidoc XRS imager with Quantity One software (Bio-Rad).

3. Results and discussion

3.1. Rapamycin-resistant ERα Ser167 phosphorylation in cells growing in serum-supplemented media

To evaluate the relative contribution of the MAPK and S6K1 signaling pathways to phosphorylation of ERα Ser167, MCF7 cells growing in 10% FBS were treated with the mTORC1 inhibitor rapamycin and MEK inhibitor U0126. As shown in Fig. 2A, treatment with rapamycin severely reduced Ser167 phosphorylation, however, some rapamycin-insensitive phosphorylation remained. While treatment with U0126 only slightly reduced phosphorylation levels, treatment with a combination of rapamycin and U0126 resulted in complete inhibition of Ser167 phosphorylation. This indicated that there exists a rapamycin-insensitive MAPK input into Ser167 phosphorylation.

3.2. Acute mitogenic stimulation of ERα Ser167 phosphorylation reveals MAPK and mTORC1/S6K1 inputs

To dissect the signaling inputs into ERα Ser167 phosphorylation, we interrogated the sensitivity of Ser167 phosphorylation to acute mitogenic stimulation. We deprived MCF7 cells of serum, and acutely stimulated them insulin, which preferentially activates the mTORC1/S6K1 pathway, or the phorbol ester phorbol 12-myristate 13-acetate (PMA), which activates both the mTORC1/S6K1 and MAPK pathways. We also investigated the effect of rapamycin and U0126 inhibitors on insulin- and PMA-stimulated ERα Ser167 phosphorylation. As shown in Fig. 2B, 30 min treatment with insu-