Identification of Novel Modulators of mTORC2 Activity

Research Plan

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a) Rationale

The mammalian target of rapamycin (mTOR) is a conserved protein kinase in all eukaryotes that acts as a master regulator of homeostasis. mTOR uses environmental inputs, including growth factors, energy, and nutrients to regulate many cellular processes such as cell growth and metabolism. This kinase is found in two structurally and functionally distinct complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of mTOR, Raptor, GβL, and PRAS40. The activity of mTORC1 is sensitive to inhibition by rapamycin; a drug that is currently in use as an immunosuppressant and being studied as a potential cancer therapeutic. mTORC1 plays a central role in increasing production of proteins, lipids, and nucleotides, while suppressing autophagy to allow a cell to grow and divide. While mTORC1 regulates cell growth and metabolism, mTORC2 controls proliferation and survival. mTORC2 is composed of mTOR, Rictor, GβL, and Sin 1 and Mutations and abnormal amplifications of these core components are the main factors for hyperactivation, which is commonly seen in many types of human cancers. It is thought to assemble at or near the plasma membrane and responds mainly to growth factors, such as insulin which then phosphorylates AGC kinase proteins, including Akt. The most crucial role of mTORC2 is most likely the phosphorylation and activation of Akt which promotes cell survival, proliferation, and growth therefore having an important role in cancer. However, the mechanism underlying this activation remains unknown.

b) Hypothesis/Goals/Expected Outcomes

The first aim of this project is to determine the optimal insulin treatments conditions required for robust mTORC2 activation in multiple cell lines. The cell lines being used include, HEK293 (human embryonic kidney), HeLa (cervical cancer), U20S (osteosarcoma), and A549 (lung cancer), these will all be treated with insulin for various times. We will follow mTORC2 activation by western blot of whole cell lysates using phosphorylated Akt as a maker of mTORC2 activity. Optimal treatment conditions will then be used in Aim 2. The second Aim and the ultimate goal of this project is to identify and characterize novel activators of mTORC2 which helps us understand how mTORC2 exactly becomes activated. Here, we will use in-cell chemical cross-linking, immunoprecipitation and mass-spectrometry. Our cell lines will be treated with and without insulin using the optimal conditions from Aim 1 and proteins will be chemically cross-linked within cells. mTORC2 will then be immunoprecipitated from cell lysates using an antibody specific towards the Rictor subunit. Immunoprecipitated material was digested while on beads using trypsin and peptides from interacting proteins will be identified via mass-spectrometry (in collaboration with the MSK mass-spectrometry core). Statistical analysis will

be performed to identify proteins that are significantly enriched or depleted upon activation of mTORC2. Gene ontology and bioinformatic analyses were conducted and used to develop a list of candidates mTORC2 activators. Preliminary results from the mentor's lab suggested that the optimal time for mTORC2 activation in the cell lines would be around 60 - 90 minutes. We can also expect many unidentified proteins to be interacting with mTORC2.

c) Procedure, Risk and Safety, Data Analysis

I. Procedure:

Cell Culture and Insulin Treatment: HEK293, HeLa, U2OS, A549 cells will be cultured in DMEM with 10% FBS and 1X Penicillin-Streptomycin. HEK 293 cells stably expressing FLAG-tagged mTORC2 will be grown in suspension with Freestyle 293 media. For mTORC2 activation assays, cells will be treated with 4μg/mL of human insulin for indicated time points.

Immunoblot Analysis: Cells will be detached from plates by scraping and pelleted by centrifugation. Cells will be disrupted by sonication in lysis buffer composed of 25mM Tris pH 7.5, 150mM NaCl, 0.2% Triton- X100, protease and phosphatase inhibitor cocktails. Lysates will be clarified by centrifugation protein concentrations were determined by Bradford assay. 10μg of protein will be loaded per condition and proteins were separated by SDS-PAGE. Proteins will be transferred to a PVDF membrane, blocked in 5% milk in TBST buffer for 30 minutes at room temperature, incubated with primary antibodies for 1 hour at room temperature, washed 3X for 10 minutes in TBST, incubated with HRP- conjugated secondary antibody for 1 hour at room temperature, and washed again 3X for 10 minutes in TBST buffer. Blots will be exposed to substrate and image on CCD imager. Bands intensities will be quantified in ImageJ software.

Immunoprecipitation and Mass Spectrometry Analysis: Lysates from ~10e6 cells will be rotated with 10μL of anti-FLAG sepharose for 1 hour at 4℃. Beads will be then transferred from Eppendorf tubes to a 96 well filter plate and pelleted by centrifugation. Beads will be washed five times in 500μL of wash buffer (25mM HEPES pH 7.5 and 150mM NaCl) using a vacuum apparatus. Proteins will be digested by incubating washed resin with MS-grade trypsin overnight at 37℃, and peptides were subject to tandem-mass tag labeling and identified by mass-spectrometry.

II. Risks and Safety

1) Human Subjects: N/A
2) Vertebrate Animals: N/A
3) Potentially Hazardous Biological Agents (PHBA): Established human lines will be used. HEK293 (human embryonic kidney), HeLa (cervical cancer), U20S (osteosarcoma), and A549 (lung cancer) will be provided from the frozen stocks

at Memorial Sloan Kettering. These cells will be handed and cultured under proper supervision. Appropriate safety equipment such as gloves, goggles, and lab coats will be used. HEK293, HeLa, U20S, and A549 cells require biosafety level two practices and containment facilities. Personal safety equipment should be worn when handling.

4) Hazardous Chemicals/Activities/Devices:

Dulbecco's modified eagle medium (DMEM):
It may cause irritation to skin, eyes, and respiratory tract if inhaled. In case of contact with eyes, one must flush thoroughly with water and seek medical assistance. If skin irritation occurs, the affected area must be washed with large amounts of soap and water. If ingested, one must wash their mouth with water and seek medical assistance.

1X Penicillin-Streptomycin:
It may cause allergic skin reaction and is harmful if swallowed. One should wear protective gloves, eye protection, face protection, and protective clothing.

10% Fetal bovine serum (FBS):
It may cause irritation to eyes, skin, or if it's inhaled or swallowed. One should use proper eye protection and appropriate clothing when handling.

Tris-HCl Stock Solution (pH 7.5):
It may cause irritation when it comes in contact with the skin and eyes. One should use proper eye protection and appropriate clothing when handling. If it's on one's skin they should wash with plenty of soap and water. In case of it coming in contact with one's eyes, they should wash with water for several minutes.

Triton X-100:
It may cause eye and skin irritation and poisoning if ingested. One should wear protective gloves, clothing, eye, and face protection.

Protease:
It may cause eye and skin irritation if it comes in contact. One should wash face, hands and any exposed skin thoroughly after handling.

Phosphatase:

Can cause irritation to eyes, skin, and respiratory tract. If it comes in contact with skin or eyes wash affected area or eyes thoroughly with water. If ingested or inhaled seek medical attention.

TBST buffer:
It may cause skin, eye and respiratory irritation. One should wear protective gloves, eye, face, and clothing protection. One should wash face, hands and any exposed skin thoroughly after handling.

HRP- conjugated secondary antibody:
It may cause skin, eye, respiratory tract irritation. Wash hands and face after handling. If eye and skin irritation occurs/persists, seek medical attention.

Anti-FLAG sepharose:
Flammable liquid and vapor and should be kept from sources of ignition. One should wear protective gloves and eye protection. If on skin take off immediately all contaminated clothing.

HEPES pH 7.5:
It may cause irritation to the eyes, skin and respiratory tract if inhaled. One should wear appropriate protective clothing and wash the affected area if it comes in contact with the skin.

MS-grade trypsin:
It can cause skin irritation and serious eye irritation. Breathing difficulties, allergy, or asthma symptoms may occur if inhaled. One should wear protective gloves, goggles, and clothing. Wash face, hands, and any exposed skin thoroughly with water after handling.

III. Data Analysis

1) Immunoprecipitation:
Using anti- FLAG sepharose, mTORC2 will be immunoprecipitated from FLAG-tagged HEK293 cells. Immunoprecipitated material will be digested while on beads using trypsin and any interacting proteins will be identified by mass-spectrometry

2) Immunoblot Analysis:
Using a CCD imager, protein bands will be visualized using enhanced chemiluminescent method (ECL) and analyzed based on their molecular weights and intensities. With the ImageJ software, band intensities will be quantified and compared.

d) Bibliography

1. Sabatini et al. (1994). RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell, Volume 78, Issue 1, Pg. 35-43.

2. Oh et al. (2011). mTOR complex 2 signaling and functions. Cell Cycle, Volume 10, Issue 14, Pg. 2305-2316.

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4. Gan et al. (2011). Evidence for Direct Activation of mTORC2 Kinase Activity by Phosphatidylinositol 3,4,5 - Trisphosphate. Program in Vascular Biology and Therapeutics.

5. Saxton et al. (2017). mTOR Signaling in Growth, Metabolism, and Disease. Whitehead Institute for Biomedical Research.

No Appendices EXIST