Secreted IGFBP5 Mediates mTORC1-Dependent Feedback Inhibition of IGF1 Signaling

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Abstract

By coupling multi-dimensional HPLC separation with a Velos Pro Orbitrap mass spectrometer, we established a high sensitivity mass spectrometry platform for comprehensive secretomic analysis.

Introduction

We sought to identify this mTORC1-regulated, secreted protein factor(s) that has IGF-1-inhibitory activity. Mass spectrometric analysis of secreted proteins, however, is technically challenging, due to their often exceedingly low abundances.By coupling multi-dimensional HPLC separation with a Velos Pro Orbitrap mass spectrometer, we established a high sensitivity mass spectrometry platform for comprehensive secretomic analysis.

Reagents

HEPES Sigma-H4034

Acetonitrile Fluka-34967

KH_2PO_4 Sigma-P9791

KCl Sigma-P9333

Rapamycin LC Laboratories-R5000

DTT Sigma-43815

Iodoacetamide Sigma-I149

UREA Sigma-U5378

Ammonium biocarbonate Sigma-6141

Trifluoroacetic acid Sigma-T6508

Formic acid Sigma-14265

Heavy ($[^{13}C_6{}^{15}N_2]$Lys, $[^{13}C_6{}^{15}N_4]$Arg) Cambridge Isotope Labs

SILAC DMEM Thermo Scientific-88420

Dilyzed FBS Thermo Scientific-88440

Sequencing-grade trypsin Promega-V5111

Equipment

Centricon ultrafiltration unit (Millipore, MWCO = 5,000 Da)

SepPak C18 columns Waters

SCX column (Polysulfoethyl aspartamide, 4.6 mm×200mm, 5 uM particle size, 200 Ǻ pore size, PolyLC).

Reverse Phase column (in-house packed)

Agilent 1100 HPLC System

Thermo LTQ Velos Pro Orbitrap mass spectrometer

Procedure

SILAC cell culture

1. Grow TSC2-/- MEFs in light ([$^{12}C_6{}^{14}N_2$]Lys, [$^{12}C_6{}^{14}N_4$]Arg) and heavy ([$^{13}C_6{}^{15}N_2$]Lys, [$^{13}C_6{}^{15}N_4$]Arg) DMEM (Cambridge Isotope Labs), respectively.
2. Supplement both light and heavy DMEM with 10% dialyzed FBS (Invitrogen). Dialyzed FBS was used to avoid the introduction of light amino acids that are naturally present in the serum.
3. Grow the cells in the corresponding media for 7 passages, at which point perform an incorporation check.
4. To do this, isolate, lyse and digest heavy cells overnight with sequencing-grade trypsin (Promega) at a 1:100 (enzyme:substrate) ratio (see the next section for detailed description of the digestion conditions). Desalt the Peptides using SepPak C18 columns (Waters) according to manufacturer’s instructions, and then analyze by LC-MS/MS experiments on an LTQ Velos Pro Orbitrap mass spectrometer (Thermo, San Jose, CA). The incorporation rate of heavy amino acids ([$^{13}C_6{}^{15}N_2$]Lys, [$^{13}C_6{}^{15}N_4$]Arg) was found to be around 97% under these conditions.

Sample preparation for mass spectrometric analysis

1. SILAC-labled TSC2-/- MEFs were serum-deprived for 24 hours, during which cells cultured in light media were treated with 20 nM rapamycin. Conditioned media (CM) was collected, which was centrifuged at 1,500 rpm for 15 min to remove residual cells. The CM samples were further filtered by 0.45 um filters.

2. Combine the light and heavy CM at 1:1 ratio (normalized by cell lysates), which were concentrated by a Centricon ultrafiltration unit (Millipore, MWCO = 5,000 Da). Extract the proteins by methanol-chloroform precipitation, and solubilize in 8 M urea. Reduce the cysteines using 2 mM DTT, and then alkylate by adding iodoacetamide to a final concentration of 50 mM, followed by incubation in the dark for 20 min. Dilute the lysates to a final concentration of 2 M urea by addition of 100 mM Ammonium bicarbonate (pH 7.8) and then digest overnight with sequencing-grade trypsin (Promega) at a 1:100 (enzyme:substrate) ratio. Quench digestion by addition of trifluoroacetic acid to a final concentration of 0.1% and remove the precipitates by centrifugation at 4,000 rpm for 30 min. Desalt the peptides using SepPak C18 columns (Waters) according to manufacturer's instructions.
3. Fractionate the peptides by using an off-line two dimensional SCX-RP-HPLC (strong-cation exchange-reverse phase) protocol. Briefly: suspend the lyophilized peptides in 500 ul SCX buffer A (5 mM KH_2PO_4, pH 2.65, 30% acetonitrile) and inject onto a SCX column (Polysulfoethyl aspartamide, 4.6 mm×200mm, 5 uM particle size, 200 Ǻ pore size, PolyLC). Gradient was developed over 35 min ranging from 0% to 21% buffer B (5 mM KH_2PO_4, pH 2.65, 30% acetonitrile, 350 mM KCl) at a flow rate of 1 ml/min. Collect twelve fractions and lyophilize them. Desalt the peptides using SepPak C18 columns and lyophilize them.
4. In the second dimension, separate the peptides on a 75 um×15cm in-house packed RP column (Maccel 200-3-C18AQ, 3 um, 200 Å) using a gradient developed over 90 min ranging 0% to 37% buffer B (97% acetonitrile, 0.1% formic acid). Introduce the peptides directly into the mass spectrometer via a hand-pulled emitter.

Mass spectrometry analysis and data processing

Analyse the SILAC sample by LC-MS/MS experiments on an LTQ Velos Pro Orbitrap mass spectrometer

(Thermo, San Jose, CA) using a top twenty CID (collision-induced dissociation) method. Search the MS/MS spectra against a composite database of the mouse IPI protein database (Version 3.60) and its reversed complement using the Sequest algorithm. Search parameters allowed for a static modification of 57.02146 Da for Cys and a dynamic modification of oxidation (15.99491 Da) on Met, stable isotope (10.00827 Da) and (8.01420 Da) on Arg and Lys, respectively. Filter the search results to include <1% matches to the reverse data base by the linear discriminator function using parameters including Xcorr, dCN, missed cleavage, charge state (exclude 1+ peptides), mass accuracy, all heavy or light Lys and Arg, peptide length and fraction of ions matched to MS/MS spectra. Perform peptide quantification using the CoreQuant algorithm. Because serum proteins are always light, we further remove the peptides in which only the light ion is present (the signal-to-noise ratio of the heavy peptide equals to zero).

Repeat the entire SILAC experiment with isotope-switching (i.e. heavy cells treated with rapamycin).