Molecular Immunology 46 (2009) 2694–2698

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Short communication

Enhanced interaction between Hsp90 and raptor regulates mTOR signaling upon T cell activation

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ARTICLE INFO

Article history:
Received 29 May 2009
Accepted 30 May 2009
Available online 7 July 2009

Keywords:
mTOR
Raptor
Hsp90
T cell
Anergy
Rapamycin

ABSTRACT

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved kinase which plays a role in integrating environmental cues. mTOR signals via two complexes: TORC1, which contains the Regulatory Associated Protein of TOR (raptor), and TORC2, which contains the Rapamycin-insensitive Companion of TOR (rictor). The immunosuppressive/anti-cancer agent rapamycin inhibits TORC1 function by disrupting the mTOR–raptor interaction. In an effort to understand the downstream consequences of TORC1 activation in T cells we performed a proteomic analysis of raptor binding proteins. Using this approach we have identified Hsp90 as an activation-induced binding partner of raptor in T cells. Pharmacologic inhibition of Hsp90 leads to a decrease in raptor expression and TORC1 activity. Furthermore, full T cell activation during Hsp90 blockade leads to T cell tolerance in the form of anergy. Overall, our findings suggest that Hsp90 inhibitors might represent a novel means of promoting T cell tolerance.

1. Introduction

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase which has been shown to integrate environmental signals in mammalian cells (Sabatini, 2006). mTOR is activated by an array of diverse inputs including insulin, amino acids, and growth factors. mTOR signals via two signaling complexes: TORC1 contains the Regulatory Associated Protein of TOR (raptor), and TORC2, which contains the Rapamycin-insensitive Companion of TOR (rictor). Through these two complexes, mTOR integrates diverse inputs to make cellular survival decisions, such as translation initiation, ribosome biogenesis, cell cycle progression, and inhibition of apoptosis.

Rapamycin and, more recently, the rapalogues everolimus, temsirolimus, and AP23573 inhibit TORC1 signaling by blocking the association of mTOR and raptor (Chan, 2004). While many current studies are focusing on the ability of rapamycin and its analogues to inhibit tumor growth, rapamycin initially was clinically employed as an immunosuppressive agent (Abraham, 1998). It was thought that rapamycin suppressed T cell function by inhibiting proliferation. Our group and others have shown that the specific inhibition of mTOR leads to T cell anergy (Colombetti et al., 2006; Zheng et al., 2007). That is, Th1 cells given full stimulation (anti-CD3 + anti-CD28) in the presence of rapamycin will fail to produce IL-2 and proliferate upon subsequent rechallenge, even in the absence of drug.

A central question for understanding mTOR function is determining how diverse upstream signals can lead to distinct downstream functional consequences. To address this issue in T cells we undertook a proteomic approach to identify novel binding proteins for the TORC1 adaptor, raptor. We have identified Hsp90 as an activation-induced binding protein for raptor in T cells. Furthermore, we demonstrate that manipulating this interaction can regulate the consequences of T cell activation.

2. Materials and methods

2.1. Mice

5C.C7 mice (Taconic Farms, Albany, NY) were used in accordance with the Institutional Animal Care and Use Committee at Johns Hopkins University.

2.2. A.E7 T cell clone

A.E7 Th1 cells were maintained as previously described (Zheng et al., 2007). Briefly, A.E7 cells were stimulated with irradiated, syngeneic APCs (10:1 APC:T cell) and 5 μM pigeon cytochrome *c* peptide for 48 h, then expanded in murine IL-2 for 10 days prior to experiments.

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doi:10.1016/j.molimm.2009.05.185

2.3. Antibodies

Anti-raptor, anti-mTOR, anti-Hsp90, anti-rictor, anti-phospho-S6K1 (T421/S424), and anti-p70S6K were purchased from Cell Signaling Technologies (Danvers, MA).

2.4. Cytokine detection

IL-2 and IFN-γ were detected using Ready-Set-Go ELISA kits from eBioscience (San Diego, CA) per the manufacturer's instructions.

2.5. Immunoblotting

T cells were harvested by centrifugation and resuspended in ice-cold lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate (glycerol-2-phosphate), 1 mM sodium orthovanadate, 1 mM PMSF, 1× protease inhibitors (Roche, Basel, Switzerland)) and mixed at 4 °C for 30 min. Lysates were cleared of debris by high speed centrifugation and quantitated for protein by a Coomasie Blue protein assay. Equal protein mass from each condition was mixed with 4× LDS buffer (Invitrogen, Carlsbad, CA) and boiled for 10 min. Lysates were then loaded into NuPAGE gels (10% Bis–Tris, 4–12% Bis–Tris, and 3–8% Tris–acetate gels were used, Invitrogen, Carlsbad, CA) and run at 200 V for 60 min. Protein was then transferred to nitrocellulose membranes with transfer buffer (1× NuPAGE Transfer Buffer (Invitrogen), 20% methanol) at 30 V for 90 min. Membranes were blocked in 5% nonfat dry milk (NFDM) for 60 min, washed briefly with Tris-buffered saline + 0.1% Tween-20 (TBST) and probed with primary antibody at an optimized dilution in 5% bovine serum albumin in TBST overnight at 4°. The membranes were then washed with TBST three times for 5 min and probed with an appropriate secondary antibody (conjugated to HRP) at an optimized dilution in NFDM. Membranes were washed two times in TBST for 5 min, and then washed with Tris-buffered saline once for 10 min. The membranes were blotted briefly on clean, adsorbent paper and incubated with enhanced chemiluminescent substrate (Denville Scientific, Metuchen, NJ) for 1 min. Blots were wrapped tightly in plastic wrap and exposed to film.

2.6. Preparation of T cell lysates and isolation of raptor associated proteins

T cells were harvested by centrifugation and resuspended in ice-cold lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 or CHAPS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate (glycerol-2-phosphate), 1 mM sodium orthovanadate, 1 mM PMSF, 1× protease inhibitors (Roche)) and mixed at 4 °C for 30 min. Lysates were centrifuged to pellet debris and the supernatant was pre-cleared with pre-washed protein G agarose (Pierce Biotechnology, Rockford, IL) for 4 h at 4 °C. 2 μg of anti-raptor (Calbiochem, San Diego, CA) was then added to the lysate and rotated gently overnight at 4 °C. Raptor immunoprecipitates were isolated by gentle mixing with pre-washed protein G agarose at 4 °C for 4 h. Immunoprecipitates were washed four times with lysis buffer, pelleted, and resuspended in boiling 2× SDS sample buffer. After brief centrifugation, the supernatant was loaded onto a 16 cm 10% Tris–glycine gel and resolved by SDS-PAGE. Gels were washed three times for 5 min in ddH_2O and then stained with SilverQuest Staining Kit (Invitrogen) as described by the manufacturer.

2.7. In-gel digestion and extraction of raptor associated protein peptides

Silver-stained bands were excised from the gel, cut into small pieces (≈1 mm^3), and destained with 200 mM ammonium bicarbonate in 40% acetonitrile for 30 min at 37 °C. The gel pieces were then dried in a speedvac and treated with 10 mM DTT for 45 min at 55 °C in 100 mM ammonium bicarbonate to reduce disulfide bonds. Cysteines were subsequently alkylated with 55 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 30 min in the dark. After successive washes with 100 mM ammonium bicarbonate and 100% acetonitrile, gel pieces were dried in a speedvac and rehydrated in 0.4 μg trypsin in 40 mM ammonium bicarbonate/9% acetonitrile. The gel pieces were incubated overnight at 37 °C to allow complete digestion. Peptides were extracted with 50% acetonitrile in 0.1% TFA, transferred to a low retention centrifuge tube, and dried in a speedvac.

2.8. Nano-flow HPLC and mass spectrometry

Peptides obtained from tryptic-gel digests were resuspended in LCMS loading buffer (3% ACN, 0.1% formic acid), and analyzed using nano-flow LC/MS/MS on an Agilent 1100 series nano-LC system (Agilent, Santa Clara, CA) coupled to an LCQ Duo ion trap mass spectrometer (ThermoFinnigan, Thermo, Waltham, MA). Peptides were pre-concentrated on a 5 mm Zorbax C18 trap column (Agilent) and then eluted onto a 100 mm × 0.075 mm custom-packed Biobasic C18 (ThermoElectron) reversed phase capillary column connected to a laser-pulled electrospray ionization emitter tip (New Objective) at a flowrate of 300 nl/min. Peptides were eluted into the nanospray source (Proxeon, Denmark) of the LCQ using the following gradient: 0% B at 0 min, 5% B at 8 min, 45% B at 50 min, 90% B at 55 min, 90% B at 60 min (B = 0.1% formic acid in acetonitrile) at a spray voltage of 2.5 kV. The LCQ was operated in data-dependent mode using the Xcalibur software (ThermoFinnigan) in which every MS scan (400–1800 m/z) was followed by MS/MS scans (400–1800 m/z) on the three most intense ions using an isolation window of ± 1.5 Da. Ions selected for MS/MS fragmentation were dynamically excluded for 30 s.

Database searching of MS/MS data was performed using the MASCOT database search engine (Matrix Science, Boston, MA) against the human NCBI non-redundant database and allowing for one missed cleavage with trypsin digestion and variable modifications of phosphorylated serine/threonine/tyrosine, oxidation of methionine, and carbamidomethylation of cysteine. The peptide mass tolerance was set to ±2 Da with a fragment mass tolerance of ±0.8 Da. All spectra matched to peptide/protein sequences were manually validated.

3. Results and discussion

T cells given full stimulation (Signal 1 + 2) in the presence of rapamycin are rendered anergic (Fig. 1A) (Powell et al., 1999). Rapamycin inhibits TORC1 activity by blocking the interaction between mTOR and raptor. To confirm this in our system, T cells were incubated in serum-free conditions in the presence of rapamycin or the Hsp90 inhibitor 17-AAG (a derivative of geldanamycin, as a negative control) for 3 h, and then given TCR and costimulation for 3 h. Immunoprecipitation (IP) of mTOR demonstrates the mTOR–raptor interaction is inhibited by rapamycin but not by 17-AAG (Fig. 1B).

Since rapamycin promotes anergy by disrupting TORC1 signaling, we were interested in finding novel binding proteins for raptor that might be involved in regulating T cell function. To do this, we utilized a proteomic strategy involving mass spectrometry (Fig. 1C). Raptor was immunoprecipitated from lysates of either resting or hyper-activated Jurkat T cells, separated by SDS-PAGE and silver stained. Protein bands were identified that were differentially bound to raptor in the lysate from stimulated versus unstimulated cells. One band identified near 90 kDa was excised, digested with trypsin, extracted and analyzed by nanospray LCMS/MS. The