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

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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.

Keywords

mTOR; raptor; Hsp90; T cell; anergy; rapamycin

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.

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AUTHOR CONTRIBUTIONS

G.M.D. performed murine T cell experiments and wrote the paper. T.P.K. performed the raptor IPs, performed MS to identify binding partners, and wrote the paper. R.J.C. designed the proteomic strategy and provided MS facilities. J.D.P. designed experiments, oversaw research, and wrote the paper.

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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 to 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.

MATERIALS AND METHODS

Mice

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

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 μ M pigeon cytochrome c peptide for 48 h, then expanded with murine IL-2 for 10 days prior to experiments.

Antibodies

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

Cytokine detection

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

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 $\times$ protease inhibitors (Roche, Basel, Switzerland)) and mixed at 4°C for 30 minutes. Lysates were cleared of debris by high speed centrifugation and quantitated for protein by a Coomassie Blue protein assay. Equal protein mass from each condition was mixed with 4X 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 200V for 60 minutes. Protein was then transferred to nitrocellulose membranes with transfer buffer (1X NuPAGE Transfer Buffer (Invitrogen), 20% methanol) at 30V for 90 minutes. Membranes were blocked in 5% nonfat dry milk (NFDM) for 60 minutes, 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 deg. The membranes were then washed with TBST three times for 5 minutes and probed with an appropriate secondary antibody (conjugated to HRP) at an optimized dilution in NFDm. Membranes were washed two times in TBST for five minutes, and then washed with Tris-buffered saline once for 10 minutes. The membranes were blotted briefly on clean, adsorbent paper and incubated with enhanced chemiluminescent substrate (Denville Scientific, Metuchen, NJ) for 1 minute. Blots were wrapped tightly in plastic wrap and exposed to film.

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 $\times$ protease inhibitors (Roche)) and mixed at 4°C for 30 minutes. Lysates were centrifuged to pellet debris and the supernatant was pre-cleared with pre-washed protein G agarose (Pierce Biotechnology, Rockford, IL) for 4 hours at 4°C. 2 μ g 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 hours. Immunoprecipitates were washed 4 $\times$ with lysis buffer, pelleted, and resuspended in boiling 2 $\times$ 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 3 $\times$ for 5 min in ddH₂O and then stained with SilverQuest Staining Kit (Invitrogen) as described by the manufacturer.

In-gel digestion and extraction of Raptor associated protein peptides

Silver-stained bands were excised from the gel, cut into small pieces ($\approx 1 \text{ mm}^3$), 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 μ g 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.

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 5mm Zorbax C18 trap column (Agilent) and then eluted onto a 100 $\times$ 0.075mm 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 3 most intense ions using an isolation window of ± 1.5 Da. Ions selected for MS/MS fragmentation were dynamically excluded for 30s.

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.

RESULTS AND DISCUSSION

T cells given full stimulation (Signal 1 + 2) in the presence of rapamycin are rendered anergic (Figure 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 hours, and then given TCR and costimulation for 3 hours. Immunoprecipitation (IP) of mTOR demonstrates the mTOR-raptor interaction is inhibited by rapamycin but not by 17-AAG (Figure 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 (Figure 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 raw LCMS/MS data was analyzed using the MASCOT search engine against the NCBI human database and two peptide sequences were identified and matched to Hsp90, a chaperone protein necessary for the correct folding of many protein “clients” (Bishop et al., 2007).

To confirm these results, we sought to demonstrate raptor-Hsp90 interaction in primary T cells. Primary 5C.C7 T cells were stimulated with peptide and expanded in IL-2 to create previously activated Th1 cells. The cells were either rested or stimulated in serum free media for 3 hours. In the stimulated T cells, IP of raptor leads to concomitant precipitation of Hsp90 (Figure 1D). Likewise, IP of Hsp90 from lysates of activated T cells leads to the concomitant precipitation of raptor. Thus, the TORC1 adaptor protein raptor binds to Hsp90 upon T cell activation.

Hsp90 is a chaperone that plays a critical role in the proper folding of a variety of proteins key to cellular survival. Using a similar proteomic approach, Ohji and colleagues also observed an interaction between raptor and Hsp90 in HEK293 cells (Ohji et al., 2006). To further determine the role of the Hsp90-raptor interaction in T cells we employed several inhibitors of Hsp90: 17-AAG and radicicol, as well as CCT018159, a newly reported pyrazole Hsp90 inhibitor. A.E7 T cells were stimulated in the presence of rapamycin or the Hsp90 inhibitors for 16 h. As expected, rapamycin had no effect on raptor expression, but stimulation during Hsp90 blockade markedly decreased raptor protein levels, consistent with the concept that raptor is an Hsp90 client (Figure 2A). T cell activation leads to increased mTOR signaling as determined by the phosphorylation of the TORC1 substrate S6K1 (Zheng et al., 2007). Consistent with previous reports (Ohji et al., 2006) in HEK293 cells, in T cells, Hsp90 inhibitors, like rapamycin, have the ability to inhibit TORC1 activity (Figure 2A).

Next we examined the functional consequences of Hsp90 inhibition in T cells. A.E7 T cells were stimulated with anti-CD3 alone or with anti-CD3 and anti-CD28 in the presence of rapamycin or Hsp90 inhibitors. The cells were washed and rested without drug. Upon initial stimulation, all T cells given costimulation produced equivalent amounts of IL-2 (Figure 2B). Such an observation suggests that Hsp90 activity is not required for T cell activation. After

five days, the cells were rechallenged with anti-CD3 and anti-CD28. Importantly, immunoblot analysis performed prior to rechallenging the cells revealed that these cells have fully regained expression of raptor protein (Figure 2C). That is by pharmacologic means, we were able to successfully knock down raptor expression during the initial encounter with antigen. On the other hand, during the rechallenge, five days later, raptor levels returned to normal. Upon rechallenge, cells initially treated with rapamycin or Hsp90 inhibitor display a marked decrease in IL-2 production. (Figure 2D). The inability to produce IL-2 upon full rechallenge is the functional hallmark of T cell clonal anergy (Schwartz, 2003). To further confirm this anergic phenotype, we interrogated the cells for IFN- γ production as well as their ability to proliferate. As expected, cells receiving Signal 1 alone or Signal 1 + 2 with rapamycin produced less IFN- γ and proliferated less upon rechallenge. In addition, those cells initially receiving Hsp90 inhibition even in the context of costimulation also produced less IFN- γ and proliferated less (Figure 2, E & F). These data demonstrate that similar to Signal 1 alone and Signal 1+2 +rapamycin, Signal 1+2 in the presence of Hsp90 blockade induced T cell anergy. That is, five days later upon rechallenge without drug present, the cells failed to proliferate or produce IFN- γ and IL-2.

Our group and others have been interested in understanding the role of mTOR in regulating T cell activation and tolerance (Powell and Zheng, 2006). In this report we demonstrate that T cell activation results in the increased binding between Hsp90 and the TORC1 component raptor. Raptor acts as an mTOR scaffolding protein facilitating the phosphorylation of mTOR substrates (Guertin et al., 2006). We performed our proteomic approach with the intention of identifying raptor binding proteins and potential TORC1 substrates upon T cell activation. However, our data suggest that raptor is in fact a client of Hsp90 rather than Hsp90 being a substrate of mTOR. Consistent with this conclusion is the observation that the Hsp90 inhibition led to a decrease in raptor protein levels. In transformed HEK293 cells, raptor is a client of Hsp90 and that its inhibition results in a decrease in TORC1 signaling (Ohji et al., 2006). Our data show that not only does this hold true in non-transformed primary T cells, but, functionally, inhibition of Hsp90 results in anergy induction. We cannot completely rule out off-target effects of pharmacologic inhibition of Hsp90 in our system. However, our approach employed inhibitors with different mechanisms of action and facilitated the rapid return of raptor expression. Whereas knockdown of Hsp90 via RNAi might be more specific, it also may be *more* problematic, as a prolonged decrease in expression of such a vital protein could show increases in non-raptor-related events. In our system, we can control the timing of Hsp90 inhibition and remove blockade from culture enabling us to study the long term downstream consequences of TCR engagement in the absence of raptor even after raptor levels are returned to normal. In this fashion, we have defined Hsp90 inhibitors as novel pharmacologic inducers of anergy. Interestingly, Hsp90 inhibitors are currently being evaluated as anti-neoplastic agents (Bishop et al., 2007). Our data suggest that while such agents might not acutely inhibit T cell function (Figure 2B), they may induce anergy in Th1 cells. As such Hsp90 inhibitors might be incorporated into immunosuppressive regimens to treat autoimmune disease or prevent transplant rejection through promotion of T cell tolerance.

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Figure 1. Hsp90 is a binding partner of the TORC1 component raptor

(A) Rapamycin induces anergy in the presence of costimulation. A.E7 Th1 cells were stimulated overnight with α -CD3, α -CD3 and α -CD28, or α -CD3, α -CD28, and rapamycin. Cells were then washed and rested in media for five days and restimulated with α -CD3 and α -CD28. Error bars indicate S.D. Results are representative of five independent experiments. (B) Rapamycin inhibits TORC1 assembly. Cells were mock or α -CD3 + α -CD28 stimulated in the presence of rapamycin or 17-AAG, lysates were made and immunoprecipitation (IP) for mTOR was performed. Immunoblots (IB) were performed on the IPs for the presence of raptor. IBs are representative of three independent experiments. (C) Proteomic strategy for identifying TORC1 substrates. Jurkat T cells were either incubated in serum-free media (to reduce the

amount of basal mTOR activation from serum) or stimulated with 500 μ M pervanadate and 20 nM caliculin A. The cells were then lysed and subjected to an IP of raptor. The two lysates were separated by SDS-PAGE and silver stained. We then looked for protein bands 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 (top panel). The raw LCMS/MS data was analyzed using the MASCOT search engine against the NCBI human database; two peptide sequences were identified and matched to Hsp90 (bottom panel, sequence in red). (D) Hsp90 and raptor interact in a TCR-induced manner. 5C.C7 primary Th1 cells were serum-starved for 3 hours, then left unstimulated or given α -CD3 and α -CD28 for 3 hours, lysed and subjected to the IP indicated. IPs were then probed for the binding partner. IBs are representative of three independent experiments.

Figure 2. Hsp90 inhibition in T cells leads to the development of an anergic state

(A) Raptor is a client of Hsp90, which is necessary for TORC1 signaling. A.E7 Th1 cells were stimulated in the presence of rapamycin (200nM), 17-AAG (200nM), radicalcol (10μM), or CCT018159 (5μM), then probed for raptor and phospho-S6K1 by IB. rictor (the TORC2 adaptor) is included as a loading control. IBs are representative of three independent experiments. (B) A.E7 Th1 cells were stimulated with α-CD3, α-CD3 and α-CD28, or α-CD3, α-CD28, and drug overnight, after which IL-2 production was measured by ELISA from a sample of supernatant (induction). (C) Cells from B were then washed and rested in unsupplemented fresh media (no drug). After 5 days, some cells were removed and probed for raptor and rictor by IB, to confirm raptor levels have returned. (D) The remainder of the cells

were stimulated with α -CD3 and α -CD28 (rechallenge with no drug) and interrogated for IL-2 production by ELISA. Results are pooled from three independent experiments. (E) Supernatants from D were interrogated for IFN- γ production by ELISA. Results are pooled from three independent experiments. (F) Cells restimulated in D were pulsed with ^3H -thymidine and assayed for proliferation. Error bars indicate S.D. Results are representative of three independent experiments.