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Hsp70 associates with Rictor and is required for mTORC2 formation and activity

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Abstract

mTORC2 is a multiprotein kinase composed of mTOR, mLST8, PRR5, mSIN1 and Rictor. The complex is insensitive to rapamycin and has demonstrated functions controlling cell growth, motility, invasion and cytoskeletal assembly. mTORC2 is the major hydrophobic domain kinase which renders Akt fully active via phosphorylation on serine 473. We isolated Hsp70 as a putative Rictor interacting protein in a yeast two-hybrid assay and confirmed this interaction via co-immunoprecipitation and colocalization experiments. In cells expressing an antisense RNA targeting Hsp70, mTORC2 formation and activity were impaired. Moreover, in cells lacking Hsp70 expression, mTORC2 activity was inhibited following heat shock while controls demonstrated increased mTORC2 activity. These differential effects on mTORC2 activity were specific, in that mTORC1 did not demonstrate Hsp70-dependent alterations under these conditions. These data suggest that Hsp70 is a component of mTORC2 and is required for proper assembly and activity of the kinase both constitutively and following heat shock.

Keywords

Hsp70; Rictor; mTORC2; kinase activity; heat shock

Introduction

The mTOR kinase is a highly conserved, central regulator of cell growth and size [1]. Aberrant mTOR signaling is known to play a causal role in cancer development and progression, as well as in the response to mitogens, nutrients and chemotherapeutic agents [2,3]. Dysregulated mTOR signaling has also been implicated in tuberous sclerosis and lymphangiomyelomatosis [4]. mTOR is present in two functionally and structurally individual multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 [5]. The mammalian TOR complex 1 (mTORC1) contains mTOR, mLST8 and Raptor and is rapamycin sensitive. mTORC2 consists

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of Rictor, mSIN1, mLST8, PRR5 and mTOR and is rapamycin insensitive [6]. mTORC1 signaling results in the phosphorylation of its effectors p70/S6K and 4E-BP1 [2]. Activation of mTORC2 leads to phosphorylation of the Akt kinase at serine 473 resulting in Akt's full activation [7].

mTOR signaling plays a crucial role in the cellular response to various stresses [8]. mTORC1 regulates growth and protein synthesis via effects on p70S6K and 4E-BP1 and has been shown to inhibit autophagy [9]. Signaling inputs sensing the relative availability of amino acids have been demonstrated to enter the mTORC1 pathway at the level of Rheb and hVps34 [10]. Muscle contraction or fluid and sheer stresses have been shown to stimulate p70/S6K activity, while osmotic stress, heat shock, reactive oxygen intermediates and DNA damage generally decrease p70/S6K function [8]. The mechanisms by which these stimuli signal to the mTORCs are not well understood.

In an attempt to identify Rictor-binding proteins we isolated Hsp70 in a yeast two-hybrid screen. Since Akt activity has been shown to be stimulated by heat shock in a variety of cell types [11], we investigated whether the interaction of Hsp70 and Rictor played a functional role in the regulation of mTORC2 activity. Here we show that Hsp70 and Rictor associate in intact cells and that Hsp70 is a component of mTORC2. Moreover, we demonstrate that Hsp70 is required for mTORC2 formation and activity under basal conditions and following heat shock.

Materials and methods

Plasmids, cell lines and reagents

HeLa and P-19 cell lines were obtained from ATCC (Manassas, VA). P-19 control cells (P-$19_{neo\#3}$, vector only) and cells expressing an antisense RNA directed towards the Hsp70 mRNA (P-$19_{AS\text{-}Hsp70\#1}$) have been previously described [12]. Heat shock treatments were for 20 minutes at 45°C in a circulating water bath. DNA constructs composed of portions of Rictor and Hsp70 were generated by PCR and individually subcloned into pGB12 and pACT2, respectively. Antibodies to Raptor, mTOR, phospho-Ser^{473}-Akt, phospho-Thr^{389}-p70/S6K, Akt and p70/S6K were from Cell Signaling (Beverly, MA). mLST8 (GβL) antibodies were from Abcam. Hsp70 and rabbit IgG (isotype control) antibodies were from Santa Cruz Biotechnology. The Sin1 antibodies used are described elsewhere [13]. Antibodies to PRR5 were generously provided by Dr. Do-Hyung Kim (Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Twin Cities) [14].

Yeast two-hybrid screen

The yeast two-hybrid screen to isolate Rictor interacting proteins was performed using standard procedures [15]. The full-length human Rictor cDNA was cloned into pGB12 in frame with the Gal4 DNA-binding domain. This construct was used to transform AH109 cells to obtain a strain which expressed the GAL4DBD-Rictor fusion. This strain was used to screen human brain, prostate and placenta MATCHMAKER cDNA libraries in pACT2 (BD Biosciences, Clontech). Liquid β-gal assays were performed as previously described [16].

Immunoprecipitations and protein analysis

Immunoprecipitations and Western analyses were performed as previously described [13].

Immunofluorescence

HeLa cells were grown on coverslips, fixed and processed for dual immunofluorescence staining as described [17].

Results

Hsp70 interacts with Rictor in a yeast two-hybrid assay

To identify new Rictor binding proteins, we carried out a yeast two-hybrid screen using the entire human Rictor coding region as bait screened against a Gal4-activation domain library constructed from cDNAs generated from mRNAs isolated from human brain, prostate and placental cells. From this screen we identified all known Rictor interactors and additionally Hsp70 (Table 1). Of these, Hsp70 was identified in over 5% of the total clones recovered from the screen. As Akt has been identified as a substrate for mTORC2 [7] and Akt activity is known to increase following heat shock [11] we decided to investigate whether the interaction of Hsp70 with Rictor had biological significance in terms of mTORC2 activity under basal conditions and following heat shock.

Mapping of the regions involved in Hsp70 binding to Rictor

We next determined which regions of Rictor and Hsp70 were required for efficient binding as determined by the yeast two-hybrid assay. We generated a set of deletion mutants, shown in figure 1, of both Rictor and Hsp70 fused to the Gal4-DNA binding domain (DBD) or Gal4-activation domains (AD), respectively. These constructs were transformed into the yeast two hybrid strain AH109, plated on selective media to assess growth and the relative strength of the interactions were also determined by liquid β-galactosidase assays. As shown, a region within the N-terminal half of Rictor (a.a. 121–420) was required for specific binding with the full-length Hsp70-AD fusion. A region of Hsp70, within the substrate recognition domain (a.a. 168–480), was necessary to mediate strong reporter activity with full-length Rictor. Fusions of only the interacting domains within Rictor and Hsp70 to the Gal4-DBD or AD, respectively, were sufficient to mediate a robust interaction and led to growth under selective conditions and high levels of β-gal activity. These data suggested that domains within the N-terminal half of Rictor and within the substrate recognition domain of Hsp70 mediate the interaction of these two proteins.

Hsp70 associates with Rictor in mammalian cells and is specifically a component of mTORC2

To further confirm a direct association of Hsp70 with Rictor in cells, we conducted co-immunoprecipitation experiments. As shown in figure 2A, endogenous Hsp70 from HeLa cells was detectable in immunoprecipitates of Rictor, as was mTOR, but not Raptor. Similarly, in reciprocal experiments, we found that in immunoprecipitates of Hsp70, high levels of Rictor and mTOR were present, but no Raptor was detectable. Further supporting that Hsp70 was specifically associated with mTORC2 and not mTORC1, Hsp70 and Rictor were not present in Raptor immunoprecipitates. As heat shock proteins are known to dissociate from substrates under conditions of elevated ATP levels [18], we tested whether Hsp70 could be liberated from immunoprecipitated Rictor following incubation in high ATP wash buffer *in vitro*. As shown in figure 2B, Hsp70 levels were reduced in Rictor immunoprecipitates following incubation in 20 mM ATP buffer relative to immunoprecipitates incubated in non-ATP containing buffer whereas mTOR binding to Rictor was not effected. We also performed dual labeling immunofluorescence localization experiments to determine whether Rictor and Hsp70 were colocalized in cells at 37°C and following heat shock. As shown in figure 2C, Hsp70 and Rictor were colocalized diffusely throughout the cell and somewhat concentrated together at the plasma membrane (see merged image) at 37°C. However, as shown in the panels in figure 2D, both Hsp70 and Rictor were predominantly colocalized within the nucleus and peri-nuclear region following heat shock. Taken together, these data suggest that Rictor and Hsp70 associate, as components of mTORC2, in intact mammalian cells.

Downregulation of Hsp70 results in reduced mTORC2 formation and activity

To determine whether the association of Hsp70 with Rictor had functional significance we initially tested whether inhibition of Hsp70 expression had effects on mTORC2 activity. In cells overexpressing an antisense Hsp70 RNA, in which Hsp70 expression was reduced (> 95%) [12], TORCs were immunoprecipitated using anti-mTOR antibodies and the indicated components were assayed by immunoblot. As shown in figure 3A, in P-19$_{AS\text{-}Hsp70\#1}$ cells expressing the antisense RNA directed towards Hsp70, Rictor levels were markedly reduced relative to control P-19$_{neo\#3}$ cells (vector only transfectants) suggesting that Hsp70 expression was required for the mTOR-Rictor interaction. The reduction in Hsp70 expression did not significantly affect the levels of other TORC components found associated with mTOR except PRR5. PRR5 levels were reduced in mTOR immunoprecipitates in P-19$_{AS\text{-}Hsp70\#1}$ cells as compared to control cells consistent with previously reported studies demonstrating that Rictor binds PRR5 directly [14]. To assess whether mTORC2 activity was effected by inhibition of Hsp70 expression, we immunoprecipitated TORCs and determined whether they could phosphorylate an inactive GST-Akt substrate at serine 473 in an *in vitro* kinase assay. As shown in figure 3B, mTORC2 activity was markedly reduced in P-19$_{AS\text{-}Hsp70\#1}$ cells as compared to controls. These data suggest that mTORC2 formation and activity is inhibited in cells in which Hsp70 expression is downregulated.

Hsp70 is required for mTORC2/Akt signaling during heat shock

We then determined whether inhibition of Hsp70 expression affected mTORC2 formation and activity following heat shock. Extracts from P-19$_{neo\#3}$ and P-19$_{AS\text{-}Hsp\#1}$ cells were prepared prior to and following heat shock and mTORC2 complexes immunoprecipitated. These immunoprecipitates were subsequently immunoblotted for the indicated mTORC2 components shown in figure 4A. As can be seen, in P-19$_{neo\#3}$ cells Hsp70 was found associated with Rictor under basal conditions at 37°C, however a significant increase in the amount of Hsp70 bound to Rictor was observed following heat shock. In P-19$_{AS\text{-}Hsp70\#1}$ cells no detectable Hsp70 was found in mTORC2 complexes under either condition. As can also be seen, heat shock did not significantly affect the relative amounts of any of the other mTORC2 components. To determine whether the increase in Hsp70 associated with mTORC2 complexes following heat shock also correlated with an increase in mTORC2 activity, we again performed *in vitro* kinase assays of mTOR immunoprecipitates utilizing inactive GST-Akt as a substrate and monitored serine 473 phosphorylation. As shown in figure 4B, we observed a marked increase in serine 473 Akt phosphorylation following heat shock in P-19$_{neo\#3}$ cells while no significant mTORC2 activity was observed in P-19$_{AS\text{-}Hsp70\#1}$ cells prior to or following heat shock. To determine whether the Hsp70-dependent induction in activity was specific for mTORC2 following heat shock, we assessed endogenous phosphorylated serine 473 Akt levels, as well as the phosphorylation status of the mTORC1 substrate p70/S6K. As shown in figure 4C, phospho-Thr^{389} p70/S6K levels were reduced in both P-19$_{neo\#3}$ and P-19$_{AS\text{-}Hsp70\#1}$ cells following heat shock relative to levels at 37°C, while total p70/S6K levels were unchanged. However, consistent with the effects of heat shock on mTORC2 activity observed in the *in vitro* kinase assays, we observed increased levels of Ser^{473} phosphorylated Akt following heat shock in P-19$_{neo\#3}$ cells as compared to levels at 37°C. Ser^{473} phosphorylated Akt was undetectable in P-19$_{AS\text{-}Hsp70\#1}$ cells prior to and following heat shock and total Akt levels were unchanged under either condition in both cell lines. These data demonstrate that increased levels of Hsp70 associate with mTORC2 complexes following heat shock and that this increased binding correlates with elevated mTORC2 activity. Furthermore, these data suggest that Hsp70 is required to specifically enhance mTORC2 activity under heat shock conditions.

Discussion

Heat shock proteins are part of a gene regulatory program, which adapts cells to hyperthermic conditions. Here we demonstrate that Hsp70 also plays a role in the regulation of mTORC2/Akt signaling in response to heat shock. Hsp70 was identified as an interactor with Rictor in a yeast two-hybrid screen and binding mediated by discrete regions within the N-terminal half of Rictor and within the substrate recognition domain of Hsp70. The two proteins were subsequently shown to be co-immunoprecipitatable in mammalian cell extracts and colocalize under basal conditions and following heat shock. Moreover, we demonstrated that Hsp70 is specifically a component of mTORC2 and is required for formation of the complex as well as kinase activity. We additionally demonstrated that heat shock specifically induces activation of mTORC2 activity in an Hsp70-dependent manner and kinase activation correlated with elevated phospho-ser^{473} Akt levels. These data are most consistent with a model of mTORC2 activity in which Hsp70 associates with the complex via interaction with Rictor and participates in the assembly of productive mTORC2 complexes and possibly facilitates substrate recognition.

The heat shock proteins interact with a wide range of cytosolic and nuclear substrates including proteins involved in cell signaling. Hsp90 family members have been shown to regulate certain steroid hormone receptors [19,20] as well as both tyrosine and serine-threonine kinases [21–23]. Additionally, Hsp90 is known to interact with Hsp70 [24]. Thus, the observation that Hsp70 regulates mTORC2 kinase function in not unique. Others have observed heat shock protein binding to Raptor [25] and our data supports Hsp70 binding to mTOR, in that Hsp70 immunoprecipitates contained significant amounts of mTOR (figure 2A). In fact, Pearce et al., [26] have also found Hsp70 associated with endogenous Rictor in a recent proteomic analysis.

Our data also suggest a nuclear role for Hsp70 and Rictor during thermal stress. Localization studies show that Hsp70 is concentrated within the nucleus and nucleoli and may have a role in repairing heat-induced damage to nuclear functions such as mRNA splicing [27]. Additionally, it is known that constitutive expression of *Drosophila* Hsp70 accelerates recovery of nucleolar morphology after heat shock [28]. While Rictor has recently been shown to have mTORC2 independent functions [29], it is tempting to speculate that the observed colocalization of these two proteins following heat shock indicates a role for nuclear mTORC2 activity. This is supported by the observation of phosphorylated Akt within the nucleus [30].

We observed that mTORC1 activity was markedly inhibited following heat shock as phosphorylated p70/S6K levels were significantly reduced (figure 4C). This is consistent with the role of mTORC1 in regulating 4E-BP/eIF-4E-dependent protein synthesis which is repressed during heat shock [31] and with the overall inhibition of translation due to increased eIF-2α phosphorylation seen during hyperthermia [32]. However, the role of p70/S6K during heat shock is unclear. Previous reports have demonstrated that p70/S6K activity can either be repressed [33, 34] or activated [35, 36] during heat shock, possibly depending on cell-type and whether exponentially growing or G_o arrested cells were studied [36]. Our data clearly show a reduction in p70/S6K activity in P-19 embryonic carcinoma cells during heat shock while mTORC2 activity is elevated.

In conclusion, we have identified Hsp70 as a novel Rictor-binding protein and component of mTORC2 which regulates formation and activity of the kinase complex constitutively and following heat shock. Geldanamycin is currently being developed as an anti-tumor agent, supported by the observations that it inhibits Hsp90 binding to Raptor resulting in ablated mTORC1 activity [25]. Our data provide a biochemical rationale for the development of mTORC2 inhibitors possibly targeting Hsp70 or the Hsp70-Rictor interaction, particularly in

the light that mTORC2 has been shown to be hyperactive in gliomas [13] and may also act by preventing cell death.

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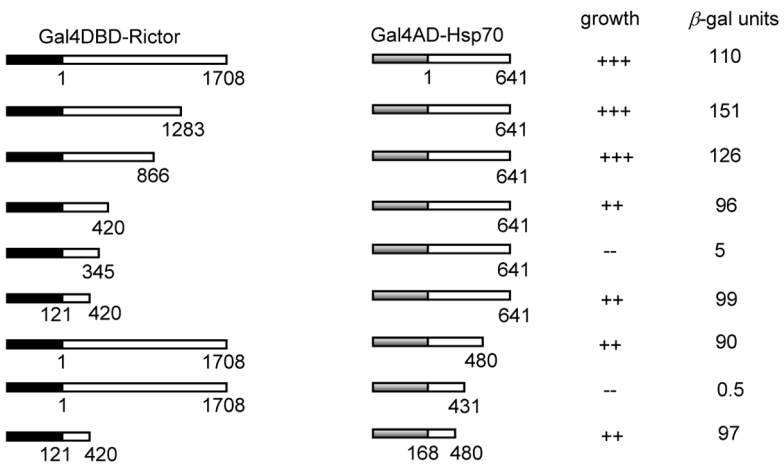

Figure 1.

Yeast two-hybrid assay mapping of interacting regions of Rictor and Hsp70. The indicated deletion mutants of Gal4DBD-Rictor or Gal4AD-Hsp70 were cotransfected into AH109 cells and plated onto selective media to determine whether an interaction between the proteins was detectable via activation of the *HIS3* reporter (+++, strong growth; ++, moderate growth; -, no growth). Colonies which grew were assayed for β-gal activity.

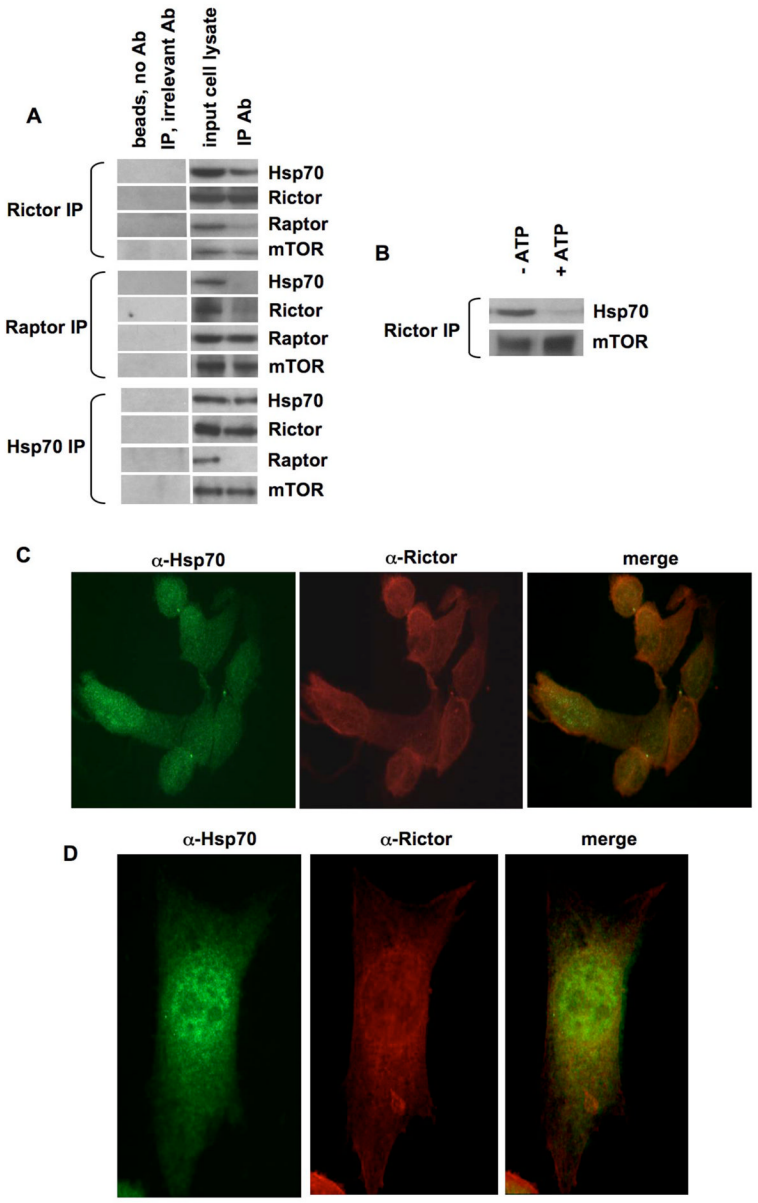

Figure 2.

Rictor interacts with Hsp70 in mammalian cells. (A) Rictor, Raptor or Hsp70 was immunoprecipitated from HeLa cells and precipitates subjected to Western analysis for the indicated proteins. Lane 1, beads, no antibody; Lane 2, immunoprecipitation with an irrelevant antibody (control IgG); Lane 3, input cell lysate; Lane 4, indicated immunoprecipitate probed with antibodies for the indicated proteins. (B) Hsp70 is liberated from Rictor in the presence of ATP. Rictor immunoprecipitates were subjected to successive washes in the absence or presence of 20 mM ATP and immunoblotted for Hsp70 and mTOR. (C) Co-localization of endogenous Hsp70 (green) and Rictor (red) in HeLa cells at 37°C and (D), following heat shock. Panels on the right show the merged composite images. Results in A-D are representative of three independent experiments.

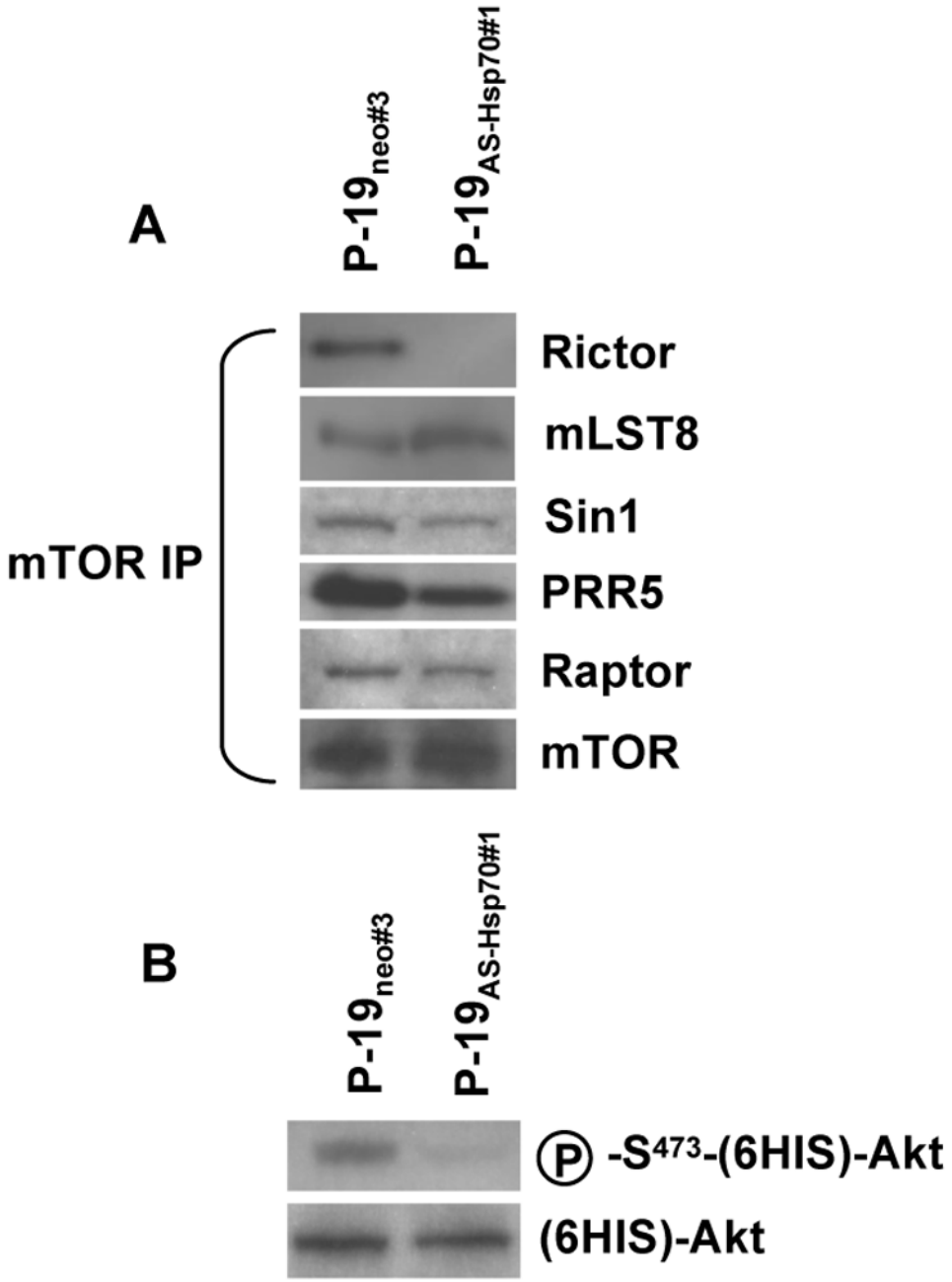

Figure 3.

Hsp70 is required for TORC2 formation and activity. (A) TORCs were immunoprecipitated from P-19 cells stably expressing an antisense RNA targeting Hsp70. These cells have previously been shown to have markedly reduced Hsp70 levels [12], in which the antisense RNA-mediated downregulation of Hsp70 was demonstrated to be specific. (B) mTORC2 *in vitro* kinase assay. TORC complexes from P-19$_{neo\#3}$ or P-19$_{AS\text{-}Hsp70\#1}$ cells were obtained using mTOR antibodies and subjected to an *in vitro* kinase assay using inactive 6HIS-tagged Akt1 as a substrate. TORC2 activity was monitored by assessing Akt1 serine 473 phosphorylation. Results in A, B were performed three times with similar results.

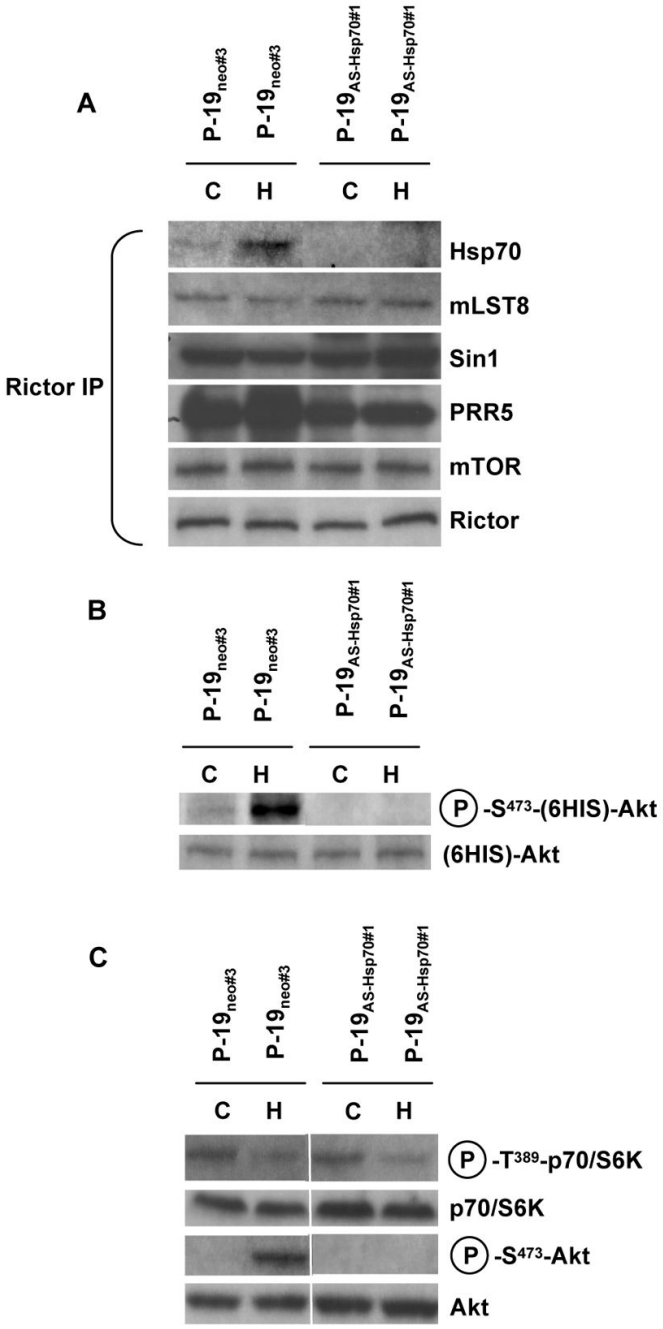

Figure 4.

mTORC2 is activated in an Hsp70-dependent manner following heat shock. (A) mTORC2 complexes were immunoprecipitated using Rictor antibodies from P-19$_{neo\#3}$ or P-19$_{AS\text{-}Hsp70\#1}$ cells under control (C, 37°C) or following heat shock (H) conditions and immunoprecipitates subjected to Western analysis for the indicated TORC2 components. (B) mTORC2 activity is increased in control P-19$_{neo\#3}$ cells and ablated in P-19$_{AS\text{-}Hsp70\#1}$ cells following heat shock. mTOR immunoprecipitates from the indicated cell lines were subjected to an *in vitro* kinase assay utilizing inactive (6HIS)-tagged Akt1 as a substrate. mTORC2 activity was monitored by assessing Akt1 serine 473 phosphorylation. (C) Hsp70-dependent TORC activity is specific for TORC2. Extracts from P-19$_{neo\#3}$ or P-19$_{AS\text{-}Hsp70\#1}$ cells under

control or following heat shock conditions were immunoblotted for endogenous phosphorylated and total p70/S6K and Akt as indicated. Results in A-C are representative of three or four independent experiments.

Table 1

Genetic interactors of Rictor in yeast two-hybrid screen

Gene	Accession No.	Cellular location	Description
Hsp70	NM_005345	nuclear/cytoplasmic	heat shock 70kDa protein 1A
mTOR	NM_004958	nuclear/cytoplasmic	Target of rapamycin kinase
mLST8	NM_022372	cytoplasmic	G protein β-subunit-like protein, GβL
mSin1	NM_024117	cytoplasmic	mitogen-activated protein kinase associated protein 1, MAPKAP1
PRR5L	NM_024841	uncharacterized	PRR5-Like ORF, Protor-2