Heat shock factor 1 (HSF1) regulates the rapid and transient expression of heat shock genes in response to stress. The transcriptional activity of HSF1 is tightly controlled, and under physiological growth conditions, the HSF1 monomer is in a heterocomplex with the molecular chaperone HSP90. Through unknown mechanisms, transcriptionally repressed HSF1-HSP90 heterocomplexes dissociate following stress, which triggers HSF1 activation and heat shock gene transcription. Using a yeast two-hybrid screening system, we have identified Ral-binding protein 1 (RalBP1) as an additional HSF1-interacting protein. We show that RalBP1 and HSF1 interact in vivo, and transient cotransfection of HSF1 and RalBP1 into hsf1−/− mouse embryonic fibroblasts represses HSP70 expression. Furthermore, transient expression of HSF1 and the constitutively active form of RalA (RalA23V), an upstream activator of the RalBP1 signaling pathway, increases the heat-inducible expression of HSP70, whereas the dominant negative form of RalBP1 (RalA28N) suppresses HSP70 expression. We further find that α-tubulin and HSP90 are also present in the RalBP1-HSF1 heterocomplexes in unstimulated cells. Upon heat shock, the Ral signaling pathway is activated, and the resulting RalGTP binds RalBP1. Concurrently, HSF1 is activated, leaves the RalBP1-HSF1-HSP90-α-tubulin heterocomplexes, and translocates into the nucleus, where it then activates transcription. In conclusion, these observations reveal that the RalGTP signal transduction pathway is critical for activation of the stress-responsive HSF1 and perhaps HSP90 molecular chaperone system.

Mammalian heat shock factor 1 (HSF1), a phosphorylated protein, regulates the stress inducibility of heat shock genes. Phosphorylation of HSF1 is indicative of its complex mode of regulation by various signaling pathways. Studies using phosphopeptide analysis of HSF1 protein as well as studies analyzing the transactivation properties of HSF1 using chimeric constructs containing GAL4-HSF1 or LexA-HSF1 have suggested that phosphorylation of serine residues Ser363 and Ser393 is likely to be involved in repression of HSF1 transcriptional activity (1–7). Mitogen-activated protein kinases and glycogen synthase kinase 3 are candidates for phosphorylating these residues. HSF1 could potentially be phosphorylated during its activation process as well, perhaps at Ser393 by calcium calmodulin protein kinase II (8). HSF1 is also found in multichaperone complexes under physiological conditions and during its repression (9–11). Specifically, HSP90 has been co-immunoprecipitated with the monomeric form of HSF1, suggesting that an HSP90-HSF1 heterocomplex may keep HSF1 in a repressed state. Disruption of this heterocomplex by stress would allow HSF1 to form trimers and acquire DNA binding capability. One likely outcome of the disruption of HSF1-HSP90 heterocomplexes during stress is the accumulation of denatured polypeptides and the ability of HSP90 to bind such denatured polypeptides (9–11). During recovery from stress, HSF1 trimers have been found in separate complexes with HSP70 and in HSP90-immunophilin (FKBPs)23 chaperone complexes (10). Heat shock factor-binding protein 1, which interacts with HSF1 trimers together with HSP70, has also been isolated (12), and this interaction appears to also be a negative regulator of HSF1 transcriptional activity.

Ral proteins are GTPases present in the plasma membrane and cytoplasmic vesicles and become biologically active through exchange of GDP for GTP (13–17). Epidermal growth factor, other receptor tyrosine kinases, and G protein-coupled receptor-induced Ral activation is dependent on Ras activation, suggesting that Ras guanine nucleotide exchange factors can also function as Ras effector molecules (17–22). Ras-dependent activation of Ral functions in parallel to the Ras-Raf-Mek-extracellular signal-regulated kinase pathway in a number of cell types (15, 17, 18, 20). In addition, Ras can be activated by a Ras-independent pathway that involves a phospholipase C-mediated increase in intracellular Ca2+ (19, 20, 23). Ras-independent activation of Ras signaling via stimulation of formin-Met-Leu-Phe receptor and dissociation of Ras-GDS from β-arrestin has been shown to cause cytoskeletal rearrangement (24). The physiological consequence of RalGTP signaling is unknown. However, control of transcription could be one end point of the RalGTP signaling (22, 25–32). Ral activation in response to insulin induces phosphorylation of c-Jun transcription factor through activation of Jun N-terminal kinase and cellular Srf (28). Similarly, activated Ral-GTPase leads to phosphorylation of Stat3 (22). Other reports suggest that expression of constitutive active Ras leads to activation of NF-κB and its downstream gene cyclin D1 (31). Ras-dependent Ral signaling pathway leads to phosphorylation of Forkhead transcription factor on threonine residues 447 and 451, leading to its activation. The protein kinase involved in such phosphorylation is unknown (25). Ras-dependent activation of RalGTP signaling has been implicated in cellular transformation; in addition, cells expressing constitutively active RalGTP show an enhanced growth rate and can form colonies in soft agar (15, 20).
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18, 33, 34). RalGTP has been shown to bind mammalian Sec 5, a subunit of the exocyst complex. Inhibition of RalA binding to Sec 5 prevents filopod production by tumor necrosis factor α and interleukin-1 (35, 36). Several Ral targets that provide clues in Ral guanine nucleotide exchange factor-induced signaling pathways have been identified. Ral has been shown to interact with phospholipase D and Arf, which suggests a role for Ral in phospholipase D-mediated vesicle transport and membrane trafficking (37, 38). Ral could also function in regulating the cytoskeleton through interaction with Ral-binding protein 1 (RalBP1) (22–24, 27, 39, 40). RalBP1 (also known as RLIP76 or RIP1) is a Ral effector molecule and associates in epidermal growth factor receptor complexes with the tyrosine-phosphorylated proteins POB1 and Reps1 (41–43). Reps1 can also bind to the adaptor proteins Crk and Grb2 (43). The significance of or the signaling pathway leading from the Ral-GTP-RalBP1 interaction is not known, but RalBP1 contains a weak GTPase (GAP) activity for Cdc42 and Rac GTPases in vitro (39, 44). Ral has therefore been shown to affect cellular proliferation, receptor-mediated endocytosis, Src kinase activation, and phospholipase D activation (15, 17, 37–39, 45, 46).

In these studies, we show that one pathway leading to HSF1 activation is mediated through the activation of the RalGTP signal transduction pathway. HSF1 interacts with a Ral effector molecule, RalBP1, both in vivo and in vitro. The HSF1-RalBP1 complex dissociates upon Ral activation by heat shock, since RalGTP has a high affinity for the Ral binding domain of RalBP1, thus leading to HSF1 activation. We also show that the HSF1-RalBP1 heterocomplexes contain HSP90 and α-tubulin.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment Conditions—H1299 is a human lung carcinoma cell line that is maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Generation of hsf1 null mouse embryo fibroblasts (MEFs) has been reported elsewhere.2 These cells were transformed with SV40 DNA and maintained in Dulbecco’s modified Eagle’s medium plus 10% heat-inactivated fetal calf serum.

Cultured tissue culture cells were heated at 80% confluence in a circulating water bath. To reach equivalent cellular cytotoxicity to heat shock between cells derived from human or mouse, H1299 cells were heated at 43 °C, and MEFs were heated at 42 °C.

Yeast Two-hybrid Screening—For yeast two-hybrid screening, we used a CytoTrap two-hybrid system (Stratagene, La Jolla, CA) (48). In this system, protein-protein interactions in the cytoplasm are detected through the recruitment of human Sos to the membranes of cells, resulting in the activation of the Ras pathway. The CytoTrap System uses the cdc25H yeast strain and a temperature-sensitive yeast homologue of human Sos. There are two plasmids, one encoding Sos, which can be fused to a bait, and one containing Myr, which can be fused to the cDNA library. In the pMyr plasmids, the genes are fused with the Src myristoylation signal, which targets and anchors the proteins in the plasma membrane. The cDNA library and the bait are cotransfected into yeast cells (3–105) were co-transfected with 1 μg of each plasmid using LipofectAMINE 2000 (Amersham Biosciences). Transfected DNA mixtures included 2 μg of expression plasmid DNA and, when required, carrier DNA added to a total of 4 μg. The DNA mix was added directly to 104 cells. The transformation frequency varied between 70 and 80% in all experiments as determined by immunofluorescence analysis.

In Vitro Transcription/Translation and RalBP1-HSF1 Interaction in Vitro—TNT rabbit reticulocyte cell lysate was used to transcribe and translate the FLAG-tagged RalBP1 according to the manufacturer’s instructions (Promega Corp., Madison, WI). Briefly, 1 μg of pcDNA3-FLAG-RalBP1 was used as a template. The reaction was carried out at 30 °C, and the product was radioabeled using [35S]methionine. 2 μl of labeled RalBP1 was incubated with 20 ng of purified full-length HSF1 in radiolabeled protein precipitation buffer for 1 h at 4 °C. Antibody to human HSF1 was then added to the reaction, and the mixture was incubated at 4 °C for 4 h. 30 μl of 50% solution of protein A solution was then added, and the protein A-agarose beads were fractionated on SDS-PAGE, and the gel was exposed to x-ray film (1).

Immunoprecipitation and Immunoblotting—Cells (3 × 106) were co-transfected with 1 μg of each plasmid using LipofectAMINE 2000. Cells were allowed to recover for 48 h, rinsed with PBS, and appropriately treated and harvested. Cells were lysed with radiolabeled protein precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, containing 1× mixture of protease inhibitors (Sigma)). 1 μg of cell lysate was precleared by incubating 30 μg of protein A-agarose beads at 4°C, 10 μg of mouse anti-FLAG monoclonal antibody (or other antibodies as indicated in the figure legends) was added to the precleared cell lysate and was incubated for an additional 2 h at 4°C. 50 μl of the pre-cleared A-agarose beads was added to the mixture and incubated for an additional 2 h. Protein A beads were then pelleted by spinning at 1000 × g for 1 min at 4°C and washed three times with radiolabeled protein precipitation buffer (1). Protein A-antibody-protein complexes were suspended in SDS sample buffer (2% SDS, 10% glycerol, 1 mM EDTA, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, 0.001% sodium dodecyl sulfate, and 0.1% SDS) and boiled. The samples were loaded on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was immunoblotted using one of the following primary antibodies: rabbit polyclonal antibody to HSF1 (1:1000) or HSP90α (1:5000) (StressGen; Victoria, Canada); mouse monoclonal antibody to HSF1 (1:5000) (StressGen), FLAG, and α-tubulin (Sigma); or polyclonal antibody to RalBP1 (1:5000) (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies were used, and signals were developed using the enhanced chemiluminescence (ECL) method (ECL kit; Amersham Biosciences) (1).

For immunoprecipitation of HSP90 heterocomplexes, H1299 cells

2 Zang, Y., Huang, L., Zhang, J., Moskophidis, D., and Mivechi, N. F. (2002) J. Cell. Biochem. 86, 376–393.
were transiently transfected with plasmids containing pcDNA3-Flag-RalBP1. After 24 h, groups of cells were incubated with 2 mM dithiothreitol (Sigma) for 30 min and then rinsed twice with 45 °C prewarmed PBS buffer, and twice with PBS at 25 °C. Other groups of cells were incubated at 37 °C and similarly rinsed with PBS. Cells were then lysed in buffer containing 50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton X-100, a mixture of protease inhibitors (Sigma) for 30 min. Cell lysates were centrifuged at 10,000 × g for 10 min. The protein concentration of the supernatant was estimated using a BCA protein assay kit (Pierce). 1 μg of each of the cell lysates was mixed with 40 μl of 50% solution of protein A-agarose and incubated at 4 °C for 1 h. The protein A-agarose was then centrifuged, and the precleared supernatant was incubated with 10 μg of rabbit polyclonal anti-HSP90α antibody (ProteinTech) or anti-RalBP1 antibody (Millipore) for 1 h at 4 °C. The beads were washed three times with 25 mM Tris-Cl (pH 7.5), 40 mM NaCl, 1% Nonidet P-40, 30 mM MgCl2, 1 mM dithiothreitol (50). Samples were heated at 100 °C for 1 min. 100 μl of 2× SDS sample buffer was added, and samples were heated at 100 °C for 5 min. 35 μl of the samples were fractionated on SDS-PAGE and analyzed by immunoblotting using appropriate antibodies.

Full-down Assay—Cell lysates were prepared in buffer containing 10% glycerol, 2% Nonidet P-40, 50 mM Tris-Cl (pH 7.4), 200 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 20 mM NaF, 1 mM sodium vanadate plus mixtures of protease inhibitors (Sigma), and 700 μg of protein from each sample was used. 20 μl of glutathione S-transferase (GST)-Ral binding domain (RalBD) purified according to the manufacturer’s instructions (Amersham Biosciences) for 1 h at 4 °C. The beads were washed three times with 25 mM Tris-Cl (pH 7.5), 40 mM NaCl, 1% Nonidet P-40, 30 mM MgCl2, 1 mM dithiothreitol (50). Samples were boiled in SDS-PAGE sample buffer and analyzed.

Immunofluorescent Analysis—105 cells were cultured on chamber slides for 24 h and were transiently transfected with 1 μg of pcDNA3-Flag-RalBP1 and allowed to grow for an additional 24 h. Cells were then rinsed with PBS and fixed with 4% paraformaldehyde for 30 min at 25 °C. Cells were then permeabilized with 0.1% Triton X-100, 0.1 mM sodium citrate for 2 min and then incubated with appropriate primary antibody and Texas Red- or fluorescein isothiocyanate-conjugated secondary antibodies as indicated in the figure legends. Nuclei were stained with 4,6-diamidino-2-phenylindole, and images were analyzed using fluorescent microscopy (5). Immunolocalization of HSF1 has been described previously (5).

FIG. 1. HSF1 interacts with RalBP1 in a yeast-two hybrid system. a, schematic representation of full-length HSF1 cDNA and the pSos-HSF1 bait plasmid containing the truncated HSF1 cDNA. See details of the yeast-two hybrid system under “Experimental Procedures.” b, yeast two-hybrid screen (using the CytoTrap system) showing HSF1 interaction with RalBP1. First row, plasmids containing pSos-ORF1 and RalBP1 clone that was isolated from cDNA library containing pMyr-RalBP1. Second row, plasmids containing pSos-MafB and pMyr-MafB (positive controls). Third row, plasmids containing pSos and pMyr-MafB (negative controls). Fourth row, plasmids containing pSos alone cotransfected with pMyr-RalBP1. c, schematic representation of pMyr-RalBP1 isolated by yeast two-hybrid screening (amino acids 440–655) and the full-length RalBP1, showing the location of the Rho GAP homology domain, Ral-binding region, POB1 binding region, and HSF1 binding region.

RESULTS

HSF1 Interacts with RalBP1—To understand the regulatory pathways involved in HSF1 activity, we used a yeast two-hybrid screening system to identify proteins that interact with HSF1. A portion of the HSF1 protein encoding the DNA binding, trimerization, and regulatory domains (amino acid residues 2–380) (Fig. 1a) and a human heart cDNA library were used. Several proteins were identified and sequenced. One of these showed 100% sequence homology to RalBP1 (or RLIP76). The fragment of RalBP1 that interacted with HSF1 contained amino acid residues 440–655 (Fig. 1, b and c).

RalBP1 is a RalGTP-binding protein (44). Full-length RalBP1 encodes a RhoGAP homology domain between amino acid residues 210 and 353 and a Ral binding domain between amino acid residues 403 and 499 (Fig. 1c). The fragment of RalBP1 that was isolated interacting with HSF1 encoded amino acid residues 440–655, encompassing a portion of the Ral binding and the entire POB1 binding region (41).

HSF1 Interacts with RalBP1 in Vitro and in Vivo—To confirm that HSF1 interacts with RalBP1 in vitro and in mammalian cells in vitro, we performed immunoprecipitation experiments. Full-length RalBP1 was transcribed and translated in vitro using [35S]methionine. The product was mixed with full-length human HSF1 and was immunoprecipitated with antibody to HSF1. Lane 1 in Fig. 2a shows that HSF1 interacts with RalBP1 in vitro.

HSF1 is monomeric and repressed at 37 °C and is mainly located in the cytoplasm. HSF1 is hyperphosphorylated and translocated into the nucleus once cells are exposed to a brief period of heat treatment (5, 51, 52). To examine whether HSF1 interacts with RalBP1 when cells are cultured at 37 °C or after cells are exposed to heat stress, H1299 cells were transiently transfected with FLAG-tagged, full-length RalBP1, and immunoprecipitation experiments were performed using antibody to FLAG (Fig. 2b) or antibody to HSF1 (Fig. 2c). The immunoblotting was performed using antibody to HSF1 (Fig. 2b) or anti-
body to RaPBPI (Fig. 2c). The results indicate that RaPBPI interacts with HSFI \textit{in vitro}, when cells are cultured at 37 °C, and that this interaction is greatly reduced when cells are exposed to 43 °C (Fig. 2b, and c, lanes 1 and 2). To ensure that the level of transiently transfected RaPBPI or the endogenous HSFI were similar in control or heated samples, immunoblotting of cell lysates from control and heated cells were performed using antibody to HSFI (Fig. 2b) or antibody to FLAG to detect RaPBPI (Fig. 2c).

Since a large fragment of HSFI was used in the yeast two-hybrid screening, we determined the region of HSFI that interacts with RaPBPI using HSFI deletion mutants. Pull-down experiments were performed using two GST fusion constructs of HSFI, one containing amino acid residues 2–81 (fragment encoding the DNA binding domain), and the other containing amino acid residues 2–270 (fragment encoding the DNA binding and trimerization domains) (Fig. 2d, and e). The lysates of the H1299 cells that were transiently transfected with full-length FLAG-RaPBPI were incubated with the purified GST-HSFI mutant proteins. Proteins that were pulled down were fractionated on SDS-PAGE, and immunoblotting was performed using antibody to FLAG. The results show that the minimal domain of HSFI that is required for binding to RaPBPI in vivo is its DNA binding domain (Fig. 2f). Since the GST-HSFI 2–270 appears to pull down more RaPBPI, some amino acid residues beyond the DNA binding domain of HSFI could be required for a more stable interaction between HSFI and RaPBPI.

The results shown in Fig. 1 indicated that the C-terminal portion of RaPBPI (amino acid residues 440–655) interacts with HSFI. Since the Ra binding domain (amino acid residues 403–499) that is capable of binding to RaGTP is located within this region on RaPBPI, experiments were performed to determine whether HSFI and RaGTP have common binding sites on RaPBPI. Pull-down experiments were performed using purified GST-RaHD (amino acid residues 403–499) and H1299 cell lysates containing endogenous HSFI. Immunoblots failed to detect HSFI, suggesting that the sequences encoded by the Ra binding domain alone were not sufficient to allow binding to HSFI (data not shown).

These results suggest that RaPBPI interacts with amino acid residues encoding the DNA binding domain of HSFI \textit{in vitro} and that this interaction occurs at 37 °C, but not when cells are exposed to heat stress \textit{in vivo}.

RaPBPI \textit{Is a Cytoplasmic Protein and, Together with \textalpha-Tubulin, Accumulates around the Nucleus after Heat Shock—A Ra-binding protein known as cytocentrin, which has high lev-
els of sequence homology to RalBP1, has been shown to be associated with the formation of mitotic spindles. Mitotic spindles contain microtubules and are composed of α-, β-, and γ-tubulins (53). To determine the intracellular location of RalBP1 and whether it is associated with microtubules, H1299 cells were transiently transfected with FLAG-RalBP1. After a 48-h incubation at 37 °C, cytoplasmic and nuclear fractions were isolated, and immunoblotting experiments were performed using antibody to RalBP1 and α-tubulin. In a similar fractionation experiment, we also used immunoblotting to detect HSF1. The results indicate that elevated levels of RalBP1 were found within the nuclear fraction after cells were exposed to heat shock, whereas an abundant amount of α-tubulin was found in both fractions (Fig. 3e). As predicted, HSF1 was translocated into the nuclear fraction as cells were heated at 39–43 °C. The translocation of HSF1 from cytoplasm into the nucleus coincides with its hyperphosphorylation and increase in its apparent molecular size (Fig. 3e) (5, 52).

To determine whether RalBP1 associates with α-tubulin, co-immunoprecipitation experiments were performed with H1299 cells transiently transfected with FLAG-RalBP1 using antibody to α-tubulin. Immunoprecipitated materials were analyzed by immunoblotting using antibody to FLAG-RalBP1 or α-tubulin. The results indicate that α-tubulin associates with RalBP1 at 37 °C and when cells were treated at 43 °C (Fig. 3b). There was some increase in association of α-tubulin with RalBP1 when cells were heated. To ensure that the level of transiently transfected RalBP1 was similar in control or heated samples, immunoblotting of cell lysates was performed using antibody to FLAG to detect RalBP1 (Fig. 3b).

We also performed immunofluorescence studies to visualize the location of RalBP1 and α-tubulin. Thus, H1299 cells were transiently transfected with FLAG-RalBP1, and control or heated cells were fixed and stained with antibody to FLAG-RalBP1 or α-tubulin. The results indicated that RalBP1 does not enter the nucleus after heat shock but accumulates around the nucleus together with α-tubulin (Fig. 3c). Similar analyses indicated that endogenous HSF1 is located in the both cytoplasm and nucleus under control conditions and that HSF1 is translocated into the nucleus and forms characteristic nuclear granules after heat shock (Fig. 3d) (5, 52).

The Ral Signaling Pathway Is Activated by Heat Shock, Leading to an Increase in RalGTP Binding to RalBP1—The Ral binding domain of RalBP1 associates with Rap in a GTP-dependent manner (39). To determine whether the Ral signaling pathway is activated by heat shock, leading to an increase in Ral-GTP as measured by an increase in Ral-GTP binding to the Ral binding domain of RalBP1, we performed the following experiments. H1299 cells were transiently transfected with expression constructs containing FLAG-RalA or the dominant negative form (FLAG-RalA28N). Cells were left untreated or were heated at 43 °C. Cell lysates were incubated with the purified GST-RalBD. Samples were then analyzed by immunoblotting using antibody to FLAG (Fig. 4). The results indicate that cells transfected with FLAG-RalA show no binding to GST-RalBD at 37 °C, indicating that the Ral signaling pathway is repressed under physiological conditions (Fig. 4). However, there is an increase in binding of FLAG-RalA to GST-RalBD within 10 min of exposure of cells to 43 °C. In contrast, cells expressing FLAG-RalA28N do not show any binding to RalBD at 37 °C or after cells were exposed to heat shock. To ensure that the levels of expression of transiently transfected FLAG-RalA and FLAG-RalA28N were similar in H1299 cells expressing these constructs, immunoblot experiments were performed with cell lysates from transiently transfected cells using antibody to FLAG (Fig. 4, lower panel).

These results indicate that treatment of cells with heat shock leads to activation of the Ral signal transduction pathway and conversion of RapGDP to RapGTP, which then binds to the Rap binding domain of RalBP1.
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Activation of the Ral Signaling Pathway Coincides with Increases in HSP70 Expression—Our findings suggest that RalBP1 binds to HSF1 at 37 °C. However, after exposure of the cells to heat shock, RalBP1 dissociates from HSF1 and binds to activated Ral. This allows HSF1 to then translocate into the nucleus and activate transcription. To test whether the activation of the Ral signaling pathway that leads to the RalGTP-RalBP1 interaction leads to altered HSP70 gene expression, H1299 cells or hsf1−/− MEFs were transiently transfected with expression constructs containing constitutively active Ral23V or the dominant negative RalA28N. Therefore, hsf1−/− MEFs were co-transfected with HSF1 expression vector. The hsf1−/− MEFs lack the ability to express any inducible heat shock proteins (HSPs) including HSP70 after heat shock. Reintroduction of HSF1 in hsf1−/− MEFs results in the induction of inducible HSPs in those cells that transiently express HSF1. Accumulation of HSP70 was examined after heat shock using immunoblotting. The results (Fig. 5, a and b) indicate that HSP70 expression is increased after heat shock when cells were transfected with constitutively active RalA23V and is decreased when cells were transfected with dominant negative RalA28N. These results confirm the above observation that the activation of the Ral signaling pathway by heat shock that is associated with an increase in RalGTP-RalBP1 binding leads to an increase in HSP70 expression. Furthermore, this effect is mainly due to HSF1, since cotransfection of dominant negative allele RalA28N together with HSF1 can completely abolish HSP70 induction in hsf1−/− MEFs (Fig. 5b).

To find out whether the expression of RalBP1 lowers the ability of HSF1 to induce HSP70, again we took advantage of hsf1−/− MEF. Thus, hsf1−/− MEFs were transfected with expression vectors containing HSF1 or HSF1 plus RalBP1. We then measured the ability of cells to induce HSP70 after heat shock. The results show that in cells expressing HSF1 and RalBP1, the level of HSP70 expression after heat shock was reduced compared with cells expressing only HSF1 (Fig. 6). Note that in some experiments, cells transiently transfected with HSF1 show basal levels of transcriptional activity even at 37 °C (Fig. 6, upper panel). This is perhaps due to the absence of sufficient negative regulators to repress HSF1 transcripational activity under control conditions once HSF1 is overexpressed (52). Interestingly, RalBP1 cotransfected with HSF1 reduces this residual constitutive active HSF1 as well as the levels of the heat-inducible HSP70 (Fig. 6).

Together with the data shown in Fig. 2, b and c, our results demonstrate that RalBP1 binds the repressed form of HSF1 at 37 °C. After heat shock, HSF1 is released from RalBP1 and is translocated into the nucleus, driving transcription of HSP70. The dissociation of HSF1 from RalBP1 could be due to the high affinity of RalGTP for RalBP1, which has been previously demonstrated (39), and we show here that it is also enhanced after exposure of cells to heat shock.

HSF1 and RalBP1 Are in Heterocomplexes with HSP90 and α-Tubulin in Vivo—HSF1 interacts with HSP90 in the cytoplasm, and this interaction keeps HSF1 in a repressed state (11). To investigate whether HSF1-RalBP1 heterocomplexes also contain HSP90 and perhaps α-tubulin, we performed co-immunoprecipitation experiments. H1299 cells were transiently cotransfected with FLAG-RalBP1 and HSF1. HSP90 was then immunoprecipitated using anti-HSP90 antibody, and the immunoprecipitated materials were analyzed by immunoblotting using antibody to detect RalBP1, HSF1, α-tubulin, or HSP90. The results indicate that HSF1, RalBP1, and α-tubulin can be communoprecipitated with HSP90 at 37 °C (Fig. 7). After heat shock, HSF1 dissociates from RalBP1, α-tubulin, and HSP90. Interestingly, some fraction of RalBP1 remains bound to HSP90 after heat shock in some experiments, suggesting slower release kinetics for HSP90 than for HSF1 from this complex. However, RalBP1 levels returned to that observed at 37 °C after the heated cells were allowed to recover for 4 h at 37 °C. At this recovery time, HSF1 had not yet appeared in the RalBP1-HSP90-α-tubulin heterocomplex (data not shown). We did not find HSP70/HSC70 in the same complex with RalBP1/HSF1-HSP90 (data not shown).

DISCUSSION

In this study, we show evidence that the Ral-binding protein RalBP1 interacts with HSF1 in vivo. The RalBP1-HSF1 interaction occurs at 37 °C, and RalBP1-HSF1 heterocomplexes dissociate after heat shock. We also find significantly high levels of HSP90 and α-tubulin in the RalBP1-HSF1 heterocomplexes. This extends the previous observations indicating that the monomeric, repressed form of HSF1 is in heterocomplexes with HSP90 (11) and extends a previous observation that HSF1-HSP90 heterocomplexes are located on α-tubulin that is a component of the cytoskeleton. Under stress conditions, HSP90-HSF1 heterocomplexes dissociate, perhaps because of the affinity of HSP90 for denatured polypeptides (9, 11). These results indicate that RalBP1-HSF1-HSP90-α-tubulin heterocomplexes receive signals from stresses that activate the Ral signaling pathway, which also leads to HSF1 activation. HSF1-HSP90 heterocomplexes are reported to be dynamic, and immunodepletion of HSP90 leads to activation of HSF1 (11). We have not detected HSP70/HSC70 proteins in RalBP1-HSF1-HSP90-α-tubulin complexes, a result that is also consistent with previous studies indicating that the repressed form of HSF1 interacts only with HSP90 and not with HSP70 (11). However, HSP70 interacts with the trimeric form of HSF1 together with HSP90 and multiple other co-chaperones during the HSF1 inactivation process; this interaction occurs in the nucleus (9, 10, 12).

We also show that the Ral signal transduction pathway is highly activated by heat shock. Activation of the Ral signaling pathway is associated with conversion of RalGDP to RalGTP and, because of the high affinity of RalGTP for RalBP1, the
RalBP1-HSF1-HSP90α-tubulin heterocomplexes can potentially dissociate, leading to release of HSF1 and allowing it to translocate into the nucleus. It is conceivable that RalBP1-HSF1-HSP90α-tubulin heterocomplexes also contain protein kinases, and such a kinase could be activated upon activation of the Ral signaling pathway. This protein kinase could then phosphorylate and activate HSF1. Similarly to the Ras-Raf-Mek-extracellular signal-regulating kinase signaling pathway, activation of the Ral signaling pathway could also lead to activation of an as yet unknown protein kinase cascade. Since Ral is found not only in the plasma membrane but also in membrane vesicles, and Ras-independent activation of Ral is calcium-dependent (13–17, 23), we hypothesize that activation of calcium-dependent protein kinases such as, for example, protein kinase C, protein kinase G, or calcium/calmodulin-dependent protein kinase, could phosphorylate and activate HSF1. Calcium/calmodulin-dependent protein kinase has been implicated in phosphorylation of HSF1 on serine 230, leading to its activation (8); however, more evidence is required to implicate this or any other enzyme in the pathway.

Similar to the other small GTP binding proteins Ras and Rac, no protein kinase has been discovered to be downstream of the Ral signal transduction pathway. Ral, through its interacting partners, has been implicated in multiple cellular processes such as endocytosis, actin/cytoskeletal organization, and vesicle function (17, 41, 43, 53). The significance of the RalBP1 binding to the active form of RalGTP is not understood. RalBP1 contains a weak GTPase activity toward Cdc42 and Rac, which influences the actin cytoskeleton and can modulate the Jun N-terminal kinase signaling pathway (33, 39, 40, 44, 54). The RalGTP-RalBP1 interaction represses the activity of Rac. Since Jun N-terminal kinase has been shown to be activated by heat shock (1) and it has been suggested that it also phosphorylates HSF1 and represses its transcriptional activity during its inactivation cycle (1), slowing down Rac activation and similarly slowing down activation of Jun N-terminal kinase after heat shock could perhaps provide a sufficient amount of time for a rapid activation of HSF1 and transcription of downstream genes.

**Fig. 5.** Activation of the Ral signaling pathway leads to activation of HSF1 and an increase in inducible HSP70 after heat shock. a and b, H1299 or hsf1−/− MEFs were transiently transfected with plasmids containing pcDNA3 (in the case of H1299) or pcDNA3-HSF1 (in the case of hsf1−/− MEFs) and cotransfected with plasmids containing RalA23V or RalA28N. After a 48-h incubation at 37 °C, cells were left untreated or were heated as indicated. Following 0–6 h of recovery time at 37 °C, cell lysates were prepared and analyzed by immunoblotting using antibody to inducible HSP70 or to FLAG as indicated. Antibody to FLAG shows levels of expression of transfected plasmid constructs.

**Fig. 6.** HSP70 expression is reduced in hsf1−/− MEFs expressing RalBP1. hsf1−/− MEFs were cotransfected with expression plasmids containing pcDNA3-HSF1 alone (upper panel) or pcDNA3-HSF1 and pcDNA3-RalBP1 (lower panel). After 48 h, cells were left untreated (C) or were heated at 42 °C for 30 min followed by recovery at 37 °C for 0 or 2 h. Equal amounts of cell lysates were analyzed by SDS-PAGE using antibody to the inducible HSP70 or RalBP1 or actin as indicated.

**Fig. 7.** HSF-1 interacts with RalBP1, HSP90, and α-tubulin. H1299 cells were transiently transfected with plasmids containing pcDNA3-RalBP1 and pcDNA3-HSF1. 48 h after transfection, cells were left untreated (lane 1) or were heated at 43 °C for 1 h (lane 2). Cell lysates were prepared, and HSP90 was immunoprecipitated using antibody to HSP90 (lanes 1 and 2) or no antibody (lane 3). The samples were immunoblotted using antibody to FLAG to detect RalBP1 or hemagglutinin to detect HSF1, anti-HSP90, or anti-α-tubulin.
genes followed by its subsequent inactivation by phosphorylation by Jun N-terminal kinase and perhaps other protein kinases. RalBP1 has also been shown to interact with the $\mu_2$ subunit of AP2, which is involved in endocytosis (49, 55). Therefore, active RalGTP has been suggested to stabilize association of $\mu_2$-RalBP1 with the membrane, leading to inhibition of clathrin-dependent endocytosis of certain receptors such as epidermal growth factor (49).

Interestingly, RalBP1 has a Ral binding region, and this region of RalBP1 partially overlaps with the region that binds HSF1. This suggests that Ral and HSF1 could compete for a binding site on RalBP1. However, the HSF1 binding region in RalBP1 requires a number of amino acids that extend to the POB1 binding region. The exact boundaries of the Ral binding region and POB1 binding region in RalBP1, however, has not been fully defined. POB1 (partner of RalBP1) is a protein with POB1 binding region. The exact boundaries of the Ral binding site on RalBP1. However, the HSF1 binding region in RalBP1 partially overlaps with the region that binds Ral.

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HSF-1 Interacts with Ral-binding Protein 1 in a Stress-responsive, Multiprotein Complex with HSP90 *in Vivo*

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