Evidence That Protein Phosphatase 5 Functions To Negatively Modulate the Maturation of the Hsp90-Dependent Heme-Regulated eIF2α Kinase†

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ABSTRACT: The maturation and activation of newly synthesized molecules of the heme-regulated inhibitor of protein synthesis (HRI) in reticulocytes require their functional interaction with Hsp90. In this report, we demonstrate that protein phosphatase 5 (PP5), a previously documented component of the Hsp90 chaperone machine, is physically associated with HRI maturation intermediates. The interaction of PP5 with HRI is mediated through Hsp90, as mutants of PP5 that do not bind Hsp90 do not interact with HRI. PP5 was also present in Hsp90 heterocomplexes with another Hsp90 cohort, p50^{cdc37}, and expression of newly synthesized HRI enhanced the amount of p50^{cdc37} associated with Hsp90/PP5-HRI heterocomplexes. The functional significance of the interaction of PP5 with Hsp90-HRI heterocomplexes was examined by characterizing the effects of compounds that impact PP5 activity in vitro. The protein phosphatase inhibitors okadaic acid and nodularin enhanced the kinase activity of HRI when applied during HRI maturation/activation, while the PP5 activators arachidonic and linoleic acid repressed HRI activity when applied during HRI maturation/activation. However, application of these compounds after HRI's "transformation" to an Hsp90-independent form did not similarly impact HRI's kinase activity. Furthermore, the Hsp90 inhibitor geldanamycin negated the effects of phosphatase inhibitors on HRI maturation/activation. The finding that PP5 downregulates an Hsp90-dependent process supports models for regulated Hsp90 function and describes a novel potential substrate for PP5 function in vivo.

The *h*eme-*r*egulated *i*nhibitor (HRI)[1] of protein synthesis is a protein kinase that acts in reticulocytes and reticulocyte lysates to coordinate the synthesis of globin chains with the availability of heme (reviewed in refs *1* and *2*). Under heme-deficient conditions, HRI becomes activated and phosphorylates the α-subunit of the eukaryotic translational initiation factor eIF2 at Ser51. Phosphorylation of eIF2α causes the sequestration of eIF2B, the guanine nucleotide exchange factor responsible for the recycling of eIF2, in complexes with phosphorylated eIF2. The subsequent accumulation of nonfunctional GDP-bound eIF2 ultimately shuts off initiation of protein synthesis. In addition to heme deficiency, HRI is also activated by other environmental or chemical stimuli including heat shock, oxidative stress, denatured proteins, and sulfhydryl-reactive reagents (*1*, *2*).

The activation of HRI under physiological and nonphysiological conditions is accompanied by phosphorylation events. While a preponderance of evidence supports the notion that the phosphorylation of HRI is autocatalytic (*2*−*5*), heterophosphorylation by casein kinase II has also been proposed to regulate HRI activation (*6*). Despite characterizations performed to date, the specific phosphorylation/dephosphorylation events regulating HRI function are poorly understood.

In addition to governing HRI activation, evidence indicates that phosphorylation of HRI also regulates its posttranslational maturation and that the pathways for HRI maturation and activation overlap (*4*, *7*, *8*). Newly synthesized HRI is inactive and cannot be activated by heme deficiency or treatment with *N*-ethylmaleimide. This "immature" population of HRI is physically associated with chaperone machinery containing the 90 kDa heat-inducible phosphoprotein Hsp90 (*4*, *7*, *8*). HRI molecules subsequently mature such that they become competent to activate in response to heme deficiency or treatment with *N*-ethylmaleimide. However, this "mature-competent" form of HRI continues to require physical and functional interactions with Hsp90 to maintain the kinase's competence to respond to activating stimuli (*4*). When mature-competent HRI is exposed to activating conditions (e.g., heme deficiency), HRI molecules become "transformed". Transformation is characterized by activation of HRI's kinase activity and the cessation of physical and functional interactions with Hsp90. Transformation of HRI appears to occur through its autophosphorylation, as it requires the presence of a conserved lysine in HRI's catalytic pocket (*4*). Additionally, transformed HRI shows retarded electrophoretic mobility on SDS−PAGE (*4*, *8*). The altered

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[1] Abbreviations: HRI, heme-regulated eIF2α kinase; eIF, eukaryotic initiation factor; eIF2α, α-subunit of eukaryotic initiation factor 2; Hsp90, 90 kDa heat shock protein; NEM, *N*-ethylmaleimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Ni^{2+}-NTA−agarose, Ni^{2+}-nitrilotriacetic acid coupled to agarose; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); ATA, aurintricarboxylic acid; PP, phosphoprotein phosphatase; TPR; tetratricopeptide repeat; FKBP, FK506 binding protein; DMSO; dimethyl sulfoxide; PVDF, poly(vinylidene difluoride).

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electrophoretic mobility of transformed and hyperphosphorylated HRI on SDS−PAGE is reversed by pretreatment of samples with alkaline phosphatase (*5*, *9*), indicating that the altered electrophoretic mobility of HRI is diagnostic of its altered phosphorylation status. While studies to date indicate that autophosphorylation governs HRI's Hsp90-dependent activation and transformation (*4*), HRI is multiply phosphorylated in vitro and in vivo (*2*−*5*, *7*−*9*), and the importance of individual phosphorylated residues has yet to be defined.

The composition of the Hsp90 chaperone machine associated with untransformed HRI has been partially characterized in previous studies (*4*, *7*, *8*, *10*−*13*). In addition to Hsp90, HRI−chaperone complexes can also contain Hsc70, p60/HOP, p50^{cdc37}, the immunophilin FKBP52, and the nucleotide-responsive cohort p23. Several Hsp90 cochaperones, such as FKBP52 and p60/HOP, contain tetratricopeptide repeat (TPR) motifs (reviewed in ref *14*) that mediate their interaction with Hsp90 (*15*−*20*). Regarding the p50^{cdc37}- and TPR-containing components of Hsp90 heterocomplexes, in vitro competition studies have suggested that these Hsp90 cohorts might represent mutually exclusive subunits that compete for binding to the Hsp90 chaperone "machine" (*18*, *21*, *22*). More recently, however, p50^{cdc37} and FKBP52 have been found to bind to Hsp90 simultaneously both in the basal (no kinase client) chaperone machine and in client−chaperone heterocomplexes (*13*). This co-occurrence suggests that the poorly characterized role(s) of these proteins is (are) exercised in concert during Hsp90-mediated protein folding.

Protein phosphatase 5 (PP5) is a novel component of the Hsp90 chaperone machine (*23*). PP5 has been found in all eukaryotic cells so far examined from yeast to humans (*24*) and has a cytoplasmic/nuclear localization (*25*−*30*). PP5 contains an N-terminal TPR domain that mediates its interaction with Hsp90 (*23*, *28*, *31*) and a C-terminal catalytic phosphatase domain (*25*, *32*, *33*). Limited proteolysis of PP5 has indicated that its phosphatase activity is negatively modulated by its TPR domain and a region at its C-terminus (*34*, *35*). Polyunsaturated fatty acids, such as arachidonic or linoleic acid, stimulate the phosphatase activity of purified PP5 substantially and are thought to relieve the autoinhibition of PP5 by binding to its TPR domain (*34*, *36*). Like the immunophilin FKBP52, competition studies performed in vitro suggest that the association of PP5 with Hsp90 can be mutually exclusive with regard to p50^{cdc37} and immunophilins (*22*).

The apparent ubiquitous expression of PP5 has prompted the conjecture that PP5 plays an as yet underappreciated role in the regulation of signal transduction within cells (*24*). This hypothesis is supported by the observations that, besides being present in heterocomplexes formed between Hsp90 and steroid hormone receptors (*23*, *37*), PP5 has been found to be associated with the atrial natriuretic peptide receptor (*32*), the CDC16/CDC27 subunits of the anaphase-promoting complex (APC) (*26*), the apoptosis signal-regulating kinase 1 (ASK1) (*29*), the A-subunit of phosphatase 2A (*38*), and the blue light photoreceptor hCRY2 (*39*). The potential physiological significance of these interactions is supported by the observations that PP5 overexpression appears to negatively regulate glucocorticoid-mediated growth arrest in vivo (*40*) and PP5 appears to act as a negative feedback inhibitor of ASK1 activation (*29*).

In this report, the physical and functional association of PP5 with HRI maturation/activation intermediates was characterized by biochemical and pharmacological approaches in rabbit reticulocyte lysate, lysates of cells in which HRI is naturally expressed. Data indicate that PP5 is a nonexclusive Hsp90 partner protein that associates with HRI during overlapping pathways of HRI maturation and regulation. Pharmacological characterizations indicate that this association is functionally relevant. The coexistence of PP5 and p50^{cdc37} within Hsp90 heterocomplexes suggests that physiological signals that regulate HRI transformation and activation may be modulated by the opposing actions of these two Hsp90 cochaperones on HRI.

EXPERIMENTAL PROCEDURES

Reagents. For in vitro translation in rabbit reticulocyte lysate, the *Eco*RI fragment of pCMV6 encoding rat FLAG-tagged PP5 (*23*) was ligated into the corresponding site of a modified version of the in vitro transcription vector pSP64T (*41*). For expression in *Escherichia coli*, the same *Eco*RI fragment was cloned into the *Eco*RI site of the bacterial expression vector pET-30a(+). Recombinant His-tagged FLAG-PP5 or His-tagged PP5/K97A or PP5/R101A in the pET-15b expression vector (*28*) was expressed in BL21(DE3) cells and purified under native conditions as described previously for His-tagged recombinant p50^{cdc37} (*8*) for addition into reaction mixtures. Rabbit Hsp90 was purified as described previously (*42*) and used for immunization of mice to produce polyclonal ascites anti-Hsp90 antibodies. Other antibodies utilized include mouse anti-PP5 (P75520, BD Transduction Laboratories), antigen affinity-purified mouse antibodies recognizing the His_5 epitope (anti-His-tag) (Qiagen), polyclonal ascites antibodies directed against p50^{cdc37} (*13*), and M2 anti-FLAG-tag antibody (Sigma). Rabbit reticulocyte lysate reaction mixtures were assembled, and proteins were synthesized de novo and radiolabeled via coupled transcription and translation as previously described (*4*, *7*, *8*).

Assays of the Effects of Pharmacological Agents on HRI Transformation. His-tagged HRI [(His_7)-HRI] was synthesized by coupled transcription and translation in rabbit reticulocyte lysate for 15 min followed by a 4 min pulse labeling with [^{35}S]Met (*4*, *7*, *8*). Aliquots (6 μL) of the reticulocyte lysate were subsequently transferred to heme-supplemented or heme-deficient reticulocyte lysate mixtures (44 μL) containing the initiation inhibitor aurintricarboxylic acid (ATA, 60 μM final) for HRI maturation/activation. These maturation/activation reactions contained or lacked 500 nM okadaic acid or nodularin (Calbiochem), 34 μM fostriecin (Calbiochem), or various concentrations of arachidonic acid (Cayman) or linoleic acid (Sigma) (50−600 μM) individually, as indicated in the figure legends. Control maturation/activation reactions contained equal amounts of okadaic acid-7,10,24,28-tetraacetate (Calbiochem) in DMSO as the negative control for okadaic acid, DMSO as the vehicle control for geldanamycin, water as the vehicle control for nodularin and linoleic acid, 95% ethanol as the vehicle control for arachidonic acid, and stearic acid (Sigma) in 100% methanol as the negative control for arachidonic and linoleic acid. After addition of HRI to the drug-treated lysates, the reaction mixtures were incubated at 30 °C for 1 h to allow for HRI maturation/activation.

To assay for the postmaturational effect of drug additions, (His_7)-[^{35}S]HRI was synthesized by coupled transcription and translation in reticulocyte lysate and matured/activated in heme-deficient reticulocyte lysate for 45 min as described above. The drugs were then added, and the reaction mixtures were incubated for an additional 20 min at 30 °C. Negative controls were as described above.

After maturation/activation in reticulocyte lysate, (His_7)-[^{35}S]HRI was immunoadsorbed from reaction mixtures with mouse anti-(His_5) monoclonal antibody prebound to agarose containing cross-linked anti-mouse IgG (anti-His-tag antibody resin). To assess the interaction of [^{35}S]HRI with Hsp90 and p50^{cdc37}, Hsp90 and p50^{cdc37} were immunoadsorbed with 8D3 anti-Hsp90 or anti-p50^{cdc37} antibodies as previously described (*4*, *8*). Reaction mixtures incubated with reticulocyte lysate containing no template were used as controls for nonspecific binding. Resins were washed with PIPES buffer (10 mM, pH 7.2) containing 150 mM NaCl and 50 mM NaF. Immunoadsorbed HRI was assayed for its eIF2α kinase activity as previously described (*4*, *7*, *8*), and gel mobility shifts on SDS−PAGE were used to monitor HRI transformation as previously described (*4*, *7*, *8*). Samples were analyzed by SDS−PAGE, electrotransfer to PVDF membranes, and autoradiography.

Protein−Protein Interaction Assays. To assay for the interaction of HRI with the endogenous PP5 in reticulocyte lysate, (His_7)-[^{35}S]HRI was synthesized by coupled transcription and translation in reticulocyte lysate for 30 min at 30 °C, after which lysate reactions were immunoadsorbed with anti-His-tag antibody resin. Immunoadsorptions were washed with PIPES buffer (10 mM, pH 7.2) containing 150 mM NaCl and 0.5% Tween-20. As negative controls, blank reticulocyte lysate reactions lacking DNA templates were assessed in parallel adsorptions. Proteins adsorbed or coadsorbed from control and experimental reticulocyte lysate were separated on 8% SDS−PAGE gels, transferred to PVDF membranes, and analyzed by autoradiography or by Western blotting with antibodies directed against PP5 or other chaperones as indicated.

In an alternative assay with enhanced sensitivity for the detection of the interaction of PP5 with HRI, FLAG-tagged PP5 and HRI were synthesized de novo by coupled transcription and translation and radiolabeled in separate reticulocyte lysate reactions for 30 min at 30 °C. Synthesis and labeling were arrested via addition of ATA (60 μM final) for 10 min, and equal volumes of these individual reactions were then mixed. Subsequently, the mixed reactions were incubated for 20 min at 30 °C with or without addition of pharmacological agents, followed by immunoadsorption of FLAG-tagged PP5 with M2 anti-FLAG-tag antibody resin. In the reciprocal experiment, (His_7)-HRI and FLAG-PP5 were synthesized and mixed as described above, followed by immunoadsorption of (His_7)-HRI with anti-His-tag antibody resin. Immunoadsorptions were washed with PIPES buffer (10 mM, pH 7.2) containing 0.5% Tween-20 and NaCl at concentrations indicated in the figure legends. Radiolabeled coadsorbing proteins were assessed by SDS−PAGE and autoradiography. The presence of other coadsorbed chaperones was assessed by Western blotting. Relative amounts of proteins were quantified by scanning densitometry.

To assess the interaction between HRI and PP5 mutants, aliquots of reticulocyte lysate containing [^{35}S]HRI generated by coupled transcription and translation were mixed with hemin-supplemented lysate that had been supplemented with purified recombinant His-tagged PP5, PP5K97A, or PP5/R101A. Reaction mixtures were incubated for 20 min at 30 °C, followed by immunoadsorption with anti-His-tag antibody resin. Samples were then analyzed by SDS−PAGE, Coomassie Blue staining, autoradiography, and Western blotting.

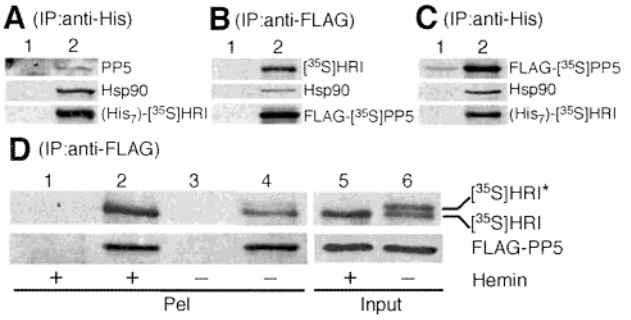

FIGURE 1: Interaction of HRI with PP5 in reticulocyte lysate. (A) Reticulocyte lysate containing (lane 2) or lacking (lane 1) plasmid encoding for (His_7)-[^{35}S]HRI was incubated for 30 min, HRI was adsorbed with anti-His-tag antibody and samples were analyzed as described under Experimental Procedures. (His_7)-[^{35}S]HRI and coadsorbed endogenous PP5 were visualized by autoradiography and Western blotting, respectively. [^{35}S]HRI (B) or (His_7)-[^{35}S]-HRI (C) and FLAG-tagged [^{35}S]PP5 were synthesized in separate reticulocyte lysate reaction mixtures for 30 min as described under Experimental Procedures. Reticulocyte lysate containing [^{35}S]HRI (B) or FLAG-tagged [^{35}S]PP5 (C) was mixed at a 1:1 ratio with lysate containing (lane 2) or lacking (lane 1) FLAG-tagged [^{35}S]-PP5 (B) or (His_7)-[^{35}S]HRI (C), respectively. After 20 min of incubation, PP5 (B) and HRI (C) were immunoadsorbed with anti-FLAG-tag and anti-His-tag antibody resin, respectively, and analyzed as described under Experimental Procedures. [^{35}S]HRI or FLAG-[^{35}S]PP5 and Hsp90 were visualized by autoradiography and Western blotting. (D) HRI was synthesized in reticulocyte lysate for 15 min as described under Experimental Procedures. Aliquots of the reactions were then diluted into 7 volumes of heme-supplemented (lanes 1, 2, and 5) or heme-deficient rabbit reticulocyte lysate (lanes 3, 4, and 6) containing (lanes 2 and 4) or lacking (lanes 1 and 3) 17 μg/mL purified recombinant FLAG-PP5. After 1 h PP5 was immunoadsorbed with M2 anti-FLAG-tag antibody resin, and samples were analyzed as described under Experimental Procedures. FLAG-PP5 and coadsorbing [^{35}S]HRI (Pel) were detected by Western blotting and autoradiography, respectively. Portions of the maturation mixtures containing the purified FLAG-PP5 were analyzed in parallel (Input). [^{35}S]HRI*, transformed form of HRI with slower electrophoretic mobility; [^{35}S]HRI, mature-competent form of HRI with faster electrophoretic mobility.

RESULTS

PP5 Is a Nonexclusive Component of HRI−Chaperone Heterocomplexes. Since PP5 has been reported to interact with Hsp90, we tested the hypothesis that PP5 occurred on heterocomplexes with the Hsp90-dependent kinase HRI. (His_7)-[^{35}S]HRI was synthesized de novo by coupled transcription and translation in heme-replete rabbit reticulocyte lysate, yielding a mixture of immature and mature-competent Hsp90-dependent molecules due to the presence of heme in translation reactions (*4*). After immunoadsorption of HRI with anti-His-tag antibody resin, coadsorbing proteins were detected by SDS−PAGE and Western blotting. As we have reported previously, Hsp90 was coadsorbed with newly synthesized (His_7)-[^{35}S]HRI (Figure 1A). In contrast, no similar (nonspecific) recovery of Hsp90 was observed in anti-

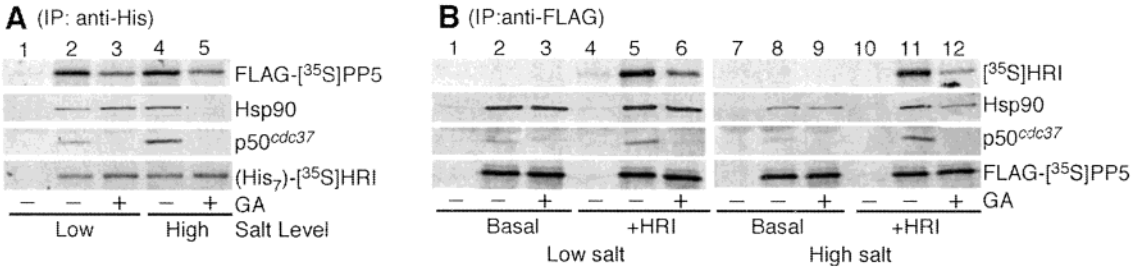

FIGURE 2: Effect of geldanamycin (GA) on the interaction of HRI with PP5 in reticulocyte lysate. (His_7)-[^{35}S]HRI was synthesized in reticulocyte lysate mixtures in the presence (+) or absence (−) of 10 μg/mL geldanamycin, and FLAG-[^{35}S]PP5 was synthesized in a separate lysate mixture for 30 min as described under Experimental Procedures. The reactions were then mixed in a 1:1 ratio with each other or with lysate lacking (His_7)-[^{35}S]HRI (A, lane 1) or lysate lacking FLAG-[^{35}S]PP5 (B, lanes 1, 4, 7, and 10) and incubated for another 20 min. Reaction mixtures were subsequently immunoabsorbed using anti-His-tag antibody resin (A) or M2 anti-FLAG-tag resin (B), followed by washing with PIPES buffers (10 mM, pH 7.2) containing no salt (A, lanes 1−3; B, lanes 1−6) or 500 mM NaCl (A, lanes 4 and 5; B, lanes 7−12), and analyzed as described under Experimental Procedures. (His_7)-[^{35}S]HRI and FLAG-[^{35}S]PP5, and endogenous Hsp90 and p50^{cdc37}, were visualized by autoradiography and Western blotting, respectively. The basal interactions of FLAG-[^{35}S]PP5 with Hsp90 and p50^{cdc37} in the absence of newly synthesized HRI are shown in panel B, lanes 2, 3, 8, and 9.

His-tag immunoadsorptions from reactions lacking (His_7)-[^{35}S]HRI. Like Hsp90, PP5 was coadsorbed with (His_7)-[^{35}S]HRI, and this adsorption was specific for reactions programmed to synthesize (His_7)-[^{35}S]HRI. These results indicated that PP5 endogenous to reticulocyte lysate occurred in heterocomplexes with HRI molecules.

To further study the interaction of HRI with PP5 in reticulcyte lysate, untagged [^{35}S]HRI or (His_7)-[^{35}S]HRI and FLAG-tagged [^{35}S]PP5 were generated in separate reticulocyte lysate reactions. The reactions were then mixed and incubated for 20 min. After incubation, PP5 (Figure 1B) or HRI (Figure 1C) was immunoadsorbed with anti-FLAG-tag or anti-His-tag antibody resin, respectively, and assayed for coabsorbing proteins. Hsp90 was specifically coadsorbed with FLAG-tagged [^{35}S]PP5 (Figure 1B, lane 2), a finding consistent with the previously described interaction of these proteins. In addition to Hsp90, [^{35}S]HRI was also coadsorbed with FLAG-tagged [^{35}S]PP5, confirming the occurrence of PP5 in heterocomplexes with HRI molecules. While a low level of nonspecific binding of PP5 to the anti-His-tag resin was noted in the reciprocal experiment (Figure 1C, lane 1), both PP5 and Hsp90 were clearly found to be coadsorbed specifically with (His_7)-[^{35}S]HRI from the reactions (Figure 1C, lane 2).

To determine whether PP5 was associated with a specific population of HRI maturation intermediates, newly synthesized [^{35}S]HRI was matured in hemin-supplemented or heme-deficient reticulocyte lysate that had been supplemented with purified recombinant FLAG-PP5. After maturational incubations, FLAG-PP5 was adsorbed with anti-FLAG antibody resin in the presence of NaF to inhibit phosphatase activity. Adsorbing/coadsorbing PP5 and HRI were detected by Western blotting and autoradiography, respectively. Incubation of HRI in heme-deficient reticulocyte lysate resulted in the transformation of approximately 55% of the HRI, as indicated by the presence of a band with retarded electrophoretic mobility relative to immature and mature-competent forms of HRI (Figure 1D, lanes 5 and 6). The fast electrophoretic mobility form (representing immature and/or mature-competent HRI molecules) was the primary form of HRI coadsorbed with PP5 from hemin-supplemented and heme-deficient lysates (Figure 1D, lanes 2 and 4). However, a proportion of HRI with retarded electrophoretic mobility (~10% of the total) was also observed to coadsorb with PP5. Previous results suggesting that Hsp90 and p50^{cdc37} interacted exclusively with immature and mature-competent (untransformed) forms of HRI (*4*, *8*) were obtained in the absence of NaF. Inclusion of NaF in buffers during anti-Hsp90 and anti-p50^{cdc37} immunoadsorptions confirmed that a small percentage of HRI with retarded electrophoretic mobility was also coadsorbed with these chaperone hetercomplexes (data not shown). These results suggest that HRI becomes phosphorylated while associated with Hsp90 heterocomplexes and that chaperone-associated phosphorylated HRI is a target for one or more phosphatases present in reticulocyte lysate.

Although in vitro competition assays have suggested that p50^{cdc37} is an exclusive Hsp90 cohort that does not coexist in heterocomplexes with FKBP52 and PP5 (*18*, *21*, *22*), our recent findings are inconsistent with this model: p50^{cdc37} and FKBP52 occur simultaneously in Hsp90 complexes (*13*). Since PP5 had similarly been postulated to be mutually exclusive with regard to p50^{cdc37} (*22*), we examined PP5-Hsp90 complexes to determine if p50^{cdc37} occurred in Hsp90 heterocomplexes with PP5. For these assays, FLAG-tagged PP5 and/or HRI were (was) synthesized, and the reactions were mixed as described above. Proteins coadsorbing with PP5 were detected by autoradiography or Western blotting. Anti-FLAG immunoadsorptions of control reactions that were not programmed to synthesize FLAG-tagged PP5 retained no detectable Hsp90, p50^{cdc37}, or [^{35}S]HRI, indicating that these adsorptions were not compromised by nonspecific binding (Figure 2B, lanes 1, 4, 7, and 10). In contrast, immunoadsorptions of reactions programmed to synthesize FLAG-tagged [^{35}S]PP5 specifically coadsorbed endogenous Hsp90 and p50^{cdc37} (Figure 2B, lanes 2 and 8). This result confirmed the previously described association of Hsp90 with PP5 and provided novel evidence for the occurrence of p50^{cdc37} in heterocomplexes containing PP5. Consistent with this finding, the occurrence of p50^{cdc37} in PP5 complexes was dramatically enriched in the presence of Hsp90-dependent HRI client (Figure 2B, lanes 5 and 11), indicating that Hsp90, PP5, and p50^{cdc37} can bind to HRI molecules concomitantly.

The PP5/HRI Interaction Is Only Partially Sensitive to Hsp90 Inhibition by Geldanamycin. Geldanamycin is a well-characterized inhibitor of Hsp90 function that acts by binding within the ATP-binding pocket of Hsp90 (*43*, *44*). This binding enforces an alternative Hsp90 conformation (*45*) that

does not support the formation of high-affinity interactions between Hsp90-p50^{cdc37} and Hsp90-dependent kinases but has no effect on the basal interaction of p50^{cdc37} with Hsp90 (*8*, *13*). This binding also prevents the Hsp90-dependent recruitment of p50^{cdc37} to kinase−Hsp90 heterocomplexes, indicating that p50^{cdc37} binding to client kinases is regulated by Hsp90's ATP-dependent conformational switching (*8*, *13*).

To test the hypothesis that PP5 binding to HRI was similarly regulated by Hsp90, reactions containing Hsp90-dependent (His_7)-[^{35}S]HRI molecules, which were generated in the presence or absence of geldanamycin, were mixed with reactions containing FLAG-[^{35}S]PP5. These reactions were incubated for 20 min, and HRI was then immunoadsorbed with anti-His-tag antibody resin. Immunoresins were washed with buffers lacking salt or with buffers containing 0.5 M NaCl, and the proteins that coadsorbed with HRI were then assessed by SDS−PAGE, autoradiography, and Western blotting.

As previously described (*8*, *13*), geldanamycin inhibited the recruitment of p50^{cdc37} to kinase−chaperone heterocomplexes and compromised the typical salt-resistant interaction of Hsp90 with its client kinase (Figure 2A). In contrast, geldanamycin only partially inhibited (~50% loss relative to untreated reactions) the association of PP5 with client HRI molecules. Furthermore, the binding of PP5 to HRI that did occur in both geldanamycin-treated and untreated reticulocyte lysate was not sensitive to high-salt washing. Importantly, PP5 remained associated with HRI after high-salt washing of immunocomplexes isolated from geldanamycin-treated reticulocyte lysate, conditions which quantitatively remove Hsp90 from complexes with client HRI molecules. Thus, PP5's association with client kinases was quite different from that observed for the p50^{cdc37}: PP5's association with HRI was not quantitatively dependent upon geldanamycin-inhibitable Hsp90 function, and PP5 remained tightly bound to HRI independent of this function. However, similar to the interaction of p50^{cdc37} with Hsp90 (*8*, *13*), geldanamycin had no impact on the magnitude of the basal chaperone−chaperone interaction of PP5 with Hsp90 (Figure 2B, lanes 2 and 8 versus lanes 3 and 9).

This novel finding was reexamined using the reciprocal approach: anti-FLAG antibody resin was used to immunoadsorb PP5 from reticulocyte lysate reactions containing or lacking FLAG-tagged PP5 (Figure 2B, +HRI). Again, PP5's association with HRI was decreased by approximately 50% in geldanamycin-treated reticulocyte lysate, but HRI remained bound to PP5 when immunocomplexes were washed with high salt (Figure 2B, lane 12), a condition which strips Hsp90 from HRI complexes (Figure 2A, lane 5). Furthermore, the presence of geldanamycin blocked the association of p50^{cdc37} with kinase−chaperone heterocomplexes (Figure 2B, lanes 6 and 12). The observation that PP5 remains bound to HRI under conditions that strip Hsp90 from immunocomplexes indicates that their interaction is not mediated solely through their common association with Hsp90.

The Interaction of PP5 with HRI Requires the Association of PP5 with Hsp90. The interaction of PP5 with Hsp90 is modulated through the interaction of conserved positively charged amino acid side chains with the conserved EEVD motif at the C-terminus of Hsp90 (*28*, *31*). To determine whether the interaction of PP5 with HRI is mediated through its association with Hsp90, we examined whether PP5 containing mutations of these positively charged amino acid residues, PP5/K97A or PP5/R101A, that do not bind Hsp90 could interact with newly synthesized HRI. Purified recombinant His-tagged wild-type PP5 or PP5 mutants were added to hemin-supplemented reticulocyte lysate and mixed with lysate containing newly synthesized HRI in the presence or absence of geldanamycin. While HRI was specifically coadsorbed from reticulocyte lysate containing wild-type PP5, little HRI was coadsorbed above the nonspecific binding control from lysate containing the PP5/K97A or PP5/R101A mutants (Figure 3). Again geldanamycin reduced the amount of HRI coadsorbing with PP5 by approximately 50%. In the presence of geldanamycin, the binding of HRI to the PP5 mutants was reduced to the level of the nonspecific background. The lack of interaction of the PP5 mutants with HRI further indicates that the binding of PP5 to HRI is specific and supports the notion that Hsp90 is responsible for targeting PP5 to maturing populations of HRI molecules.

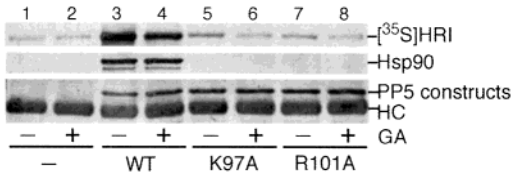

FIGURE 3: Effect of mutations that block the binding of PP5 to Hsp90 on the interaction of PP5 with HRI. [^{35}S]HRI was synthesized in reticulocyte lysate in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of 10 μg/mL geldanamycin for 30 min, as described under Experimental Procedures. Reaction mixtures containing [^{35}S]HRI were then mixed at a 1:1 ratio with normal heme-supplemented rabbit reticulocyte lysate containing (even-numbered lanes) or lacking (odd-numbered lanes) 10 μg/mL geldanamycin with no additions (lanes 1 and 2) or with the addition of 50 μg/mL purified recombinant wild-type (His_6)-PP5 (lanes 3 and 4), (His_6)-PP5/K97A mutant (lanes 5 and 6), or (His_6)-PP5/R101A mutant (lanes 7 and 8). After a 20 min incubation, reactions were absorbed anti-His-tag antibody resin and analyzed as described under Experimental Procedures. (His_6)-PP5 proteins, [^{35}S]HRI, and Hsp90 were visualized by Coomassie blue staining, autoradiography, and Western blotting, respectively. HC, antibody heavy chain.

Impact of PP5 Inhibitors: Okadaic Acid and Nodularin Induce Hyperphosphorylation of Transformed HRI. To examine the potential significance of the association of PP5 with immature/inactive HRI molecules in reticulocyte lysate, the effects of a number of phosphatase inhibitors on the Hsp90-dependent maturation, transformation, and activation of HRI were examined. (His_7)-[^{35}S]HRI was synthesized in reticulocyte lysate and subsequently matured in hemin-supplemented or heme-deficient lysate containing okadaic acid [PP5, IC_{50} = 7 nM (*46*)]. Okadaic acid had no effect on the electrophoretic mobility or kinase activity of HRI matured in hemin-supplemented lysate [wherein HRI does not undergo Hsp90-dependent transformation (not shown)]. As described previously (*4*, *7*, *8*), transfer of newly synthesized (His_7)-[^{35}S]HRI to heme-deficient reiculocyte lysate led to the generation of active HRI kinase molecules (TR), which exhibited retarded electrophoretic mobility relative to mature-competent HRI molecules (MC; Figure 4A, lane 2).

When compared to the activity of HRI that was matured and activated in control reactions, maturation and activation of HRI in reticulocyte lysate containing 500 nM okadaic acid led to the generation of HRI populations with increased kinase activity (2-fold more active than the control) (Figure

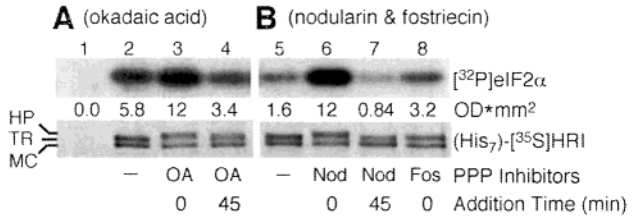

FIGURE 4: Effect of serine−threonine protein phosphatase inhibitors, okadaic acid, nodularin, and fostriecin, on HRI transformation and activation in heme-deficient reticulocyte lysate. (His_7)-[^{35}S]-HRI was synthesized in reticulocyte lysate as described under Experimental Procedures. Reactions were then transferred to 7 volumes of heme-deficient normal rabbit reticulocyte lysate mixtures supplemented with (A) 500 nM okadaic acid-7,10,24,28-tetraacetate (inactive control for okadaic acid) (−, lane 2), 500 nM okadiac acid (OA, lane 3), or no drug (lane 4) or with (B) 500 nM nodularin (Nod, lane 6), 34 μM fostriecin (Fos, lane 8), or equivalent amounts of H_2O as their vehicle controls (lanes 5 and 7). The same volume of reticulocyte lysate containing no cDNA template was similarly transferred to heme-deficient lysate as the control for any nonspecific binding of lysate protein kinase activities (A, lane 1). After 45 min of incubation, 500 nM okadaic acid (A, OA, lane 4) or 500 nM nodularin (B, Nod, lane 7) was added to the maturation mixture lacking the initial supplementation of drugs, and the incubation was continued for an additional 20 min. (His_7)-[^{35}S]-HRI was immunoadsorbed with anti-His-tag antibody resin, assayed for eIF2α kinase activity, and analyzed as described under Experimental Procedures. The amount of [^{32}P]eIF2α was quantified by scanning densitometry and expressed as optical density OD*mm^2 (numbers below the eIF2α panel). HP, hyperphosphorylated form of HRI; TR, transformed form of HRI; MC, mature-competent form of HRI.

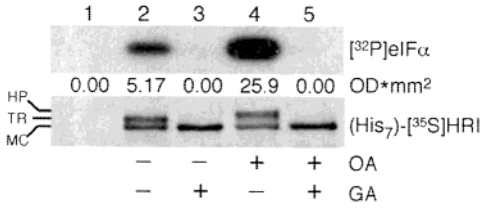

FIGURE 5: Effect of geldanamycin on the stimulation of HRI activation by okadaic acid. (His_7)-[^{35}S]HRI was synthesized in rabbit reticulocyte lysate as described under Experimental Procedures. (His_7)-[^{35}S]HRI was then transferred to 7 volumes of heme-deficient normal rabbit reticulocyte lysate mixtures supplemented with DMSO (vehicle control, lane 2), 10 μg/mL geldanamycin (GA, lane 3), 500 nM okadaic acid (OA, lane 4), or 10 μg/mL GA in combination with 500 nM okadaic acid (OA, GA, lane 5). An equivalent amount of reticulocyte lysate containing no cDNA template was similarly transferred to a heme-deficient lysate mixture containing no drug as the control for nonspecific binding of any reticulocyte lysate kinase activities (lane 1). After 60 min of incubation, (His_7)-[^{35}S]HRI was immunoadsorbed with anti-His-tag antibody resin, assayed for eIF2α kinase activity, and analyzed as described under Experimental Procedures. The amount of [^{32}P]-eIF2α was quantified by scanning densitometry of autoradiograms and expressed as optical density OD*mm^2 (numbers below the eIF2α panel). HP, hyperphosphorylated form of HRI; TR, transformed form of HRI; MC, mature-competent form of HRI.

4A, lane 3). Incubation in the presence of okadaic acid also resulted in the production of HRI molecules with electrophoretic mobilities even slower than those typical of the transformation process, suggesting that the transformed HRI had become hyperphosphorylated (HP). Consistent with previous reports indicating that these mobility shifts are due to phosphorylation events (*5*, *9*), treatment of samples with purified alkaline phosphatase generated HRI populations with fast electrophoretic mobilities similar to mature-competent HRI (not shown). Quantification of the amount of [^{35}S]HRI present in each lane confirmed that equivalent amounts of HRI were recovered from each reaction mixture. Thus, the observed increase in the phosphorylation of eIF2α represented an increase in the specific kinase activity of HRI.

Since HRI's dependence on Hsp90 chaperone machinery is conditional with respect to HRI's activation status, we next examined the impact of okadaic acid when applied to HRI populations 45 min *after* their Hsp90-dependent maturation and activation. In contrast to its application concomitant with maturation/activation, addition of okadaic acid after HRI maturation/activation did not lead to the enhanced activation of HRI's eIF2α kinase activity (Figure 4A, lane 4). In fact, addition of okadaic acid had a slight inhibitory effect on the kinase activity of preactivated HRI populations. In addition, almost no HRI with the very slow electrophoretic mobility diagnostic of HRI's hyperphosphorylation was observed when okadaic acid was added to reticulocyte lysate after most of the HRI had undergone its Hsp90-dependent transformation process. Nodularin (*47*, *48*), a water-soluble phosphatase inhibitor with a structure similar microcystin [IC_{50} for PP5 of 2.5 nm (*46*)], had effects comparable to those of okadaic acid on HRI's maturation/transformation and activity (Figure 5B, lanes 5−7).

The effect of a third phosphatase inhibitor, fostriecin, was also examined. Maturational reaction mixes were incubated in the presence of 34 μM fostriecin, a concentration which is inhibitory to PP2A (IC_{50} = 3.2 nM) but is well below its IC_{50} for PP1 (130 μM) and PP5 (700 μM) (*46*, *49*). Addition of fostriecin caused only a slight stimulation of HRI's kinase activity and had little effect on HRI's electrophoretic mobility (Figure 4B, lane 8). This result suggested that PP2A did not mediate the effects of phosphatase inhibitors on HRI maturation/activation; nonetheless, PP1, as well as PP5, remained as a candidate phosphatase.

Taken together, the experiments described in Figure 4 indicated that the specific activity of HRI was enhanced only when phosphatase inhibitors were applied concomitant with Hsp90-dependent activation/maturation events. This concomitance implied that the compounds were acting at, or upon, this stage of HRI maturation/activation. To support this conclusion, we examined the impact of preventing HRI maturation/activation by the concurrent addition of the Hsp90 inhibitor geldanamycin with okadaic acid. Consistent with the results seen in Figure 4, HRI matured/activated in the presence of okadaic acid was 5-fold more active than HRI matured in the presence of okadaic acid-7,10,24,28-tetraacetate, an inactive analogue of okadaic acid (Figure 5). However, HRI synthesized in the presence of geldanamycin was inactive irrespective of okadaic acid application. Thus, Hsp90 function was a prerequisite for the coincident stimulation of HRI kinase activity induced by okadaic acid.

Polyunsaturated Fatty Acids Inhibit HRI Transformation and Activation in a Dose-Dependent Fashion. To further test the hypothesis that PP5 function impacted the Hsp90-dependent maturation/activation of HRI, we examined the effect of arachidonic acid on this process. Arachidonic acid is a polyunsaturated fatty acid that stimulates the phosphatase activity of PP5 in vitro (*34*, *36*). Application of this compound during HRI maturation/activation led to the generation of HRI molecules that were deficient in kinase activity (Figure 6A, lanes 3−7) relative to those produced

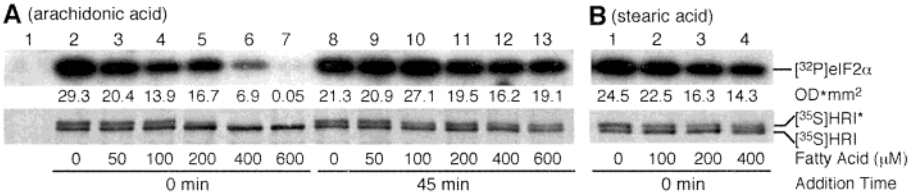

FIGURE 6: Effect of arachidonic acid (A) and stearic acid (B) on HRI transformation and activation in heme-deficient reticulocyte lysate. (His_7)-[^{35}S]HRI was synthesized in reticulocyte lysate as described under Experimental Procedures and then transferred to 7 volumes of heme-deficient normal rabbit reticulocyte lysate mixtures supplemented with 50, 100, 200, 400, or 600 μM arachidonic acid (A, lanes 3−7), an equivalent amount of 95% ethanol as the vehicle control (A, lane 2), 100, 200, or 400 μM stearic acid (B, lanes 2 to 4), or an equivalent amount of methanol as the vehicle control (B, lane 1). (His_7)-[^{35}S]HRI was also incubated in a duplicate set of heme-deficient reticulocyte lysate mixtures lacking the initial supplementation of fatty acids (A, lanes 8−13), to which 50, 100, 200, 400, or 600 μM arachidonic acid (lanes 9−13) or 95% ethanol (lane 8) was added after 45 min of maturation, followed by incubation for another 20 min. An equivalent amount of reticulocyte lysate containing no cDNA template was similarly transferred to the heme-deficient reticulocyte lysate mixture containing no drug as the control for nonspecific binding of any protein kinase activities (A, lane 1). (His_7)-[^{35}S]HRI was immunoadsorbed with anti-His-tag antibody resin, assayed for eIF2α kinase activity, and analyzed as described under Experimental Procedures. The amount of [^{32}P]eIF2α was quantified by scanning densitometry and expressed as optical density OD*mm^2 (numbers below the eIF2α panel). [^{35}S]HRI*, transformed form of HRI; [^{35}S]HRI, mature-competent form of HRI.

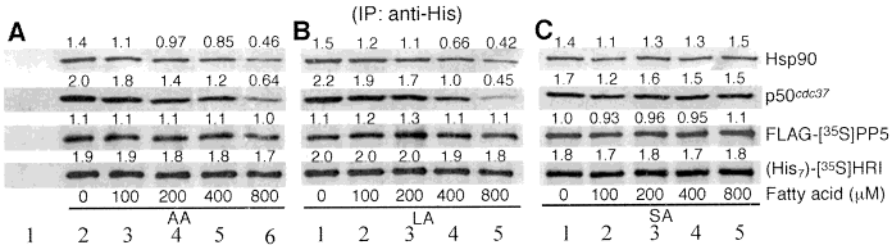

FIGURE 7: Effect of polyunsaturated fatty acids on the integrity of the HRI/Hsp90/p50^{cdc37}/PP5 heterocomplex. (His_7)-[^{35}S]HRI and FLAG-tagged [^{35}S]PP5 were synthesized in reticulocyte lysate mixtures as described under Experimental Procedures. The reactions were then mixed in a 1:1 ratio and incubated in the presence of 100, 200, 400, or 800 μM arachidonic acid (A, AA, lanes 3−6), linoleic acid (B, LA, lanes 2−5), stearic acid (C, SA, lanes 2−5), or their respective vehicle controls [A, 95% ethanol (lane 2); B, H_2O (lane 1); C, methanol (lane 1)]. The reticulocyte lysate mixture lacking the (His_7)-HRI template was similarly incubated with FLAG-[^{35}S]PP5-containing lysate as the control for nonspecific binding (A, lane 1). Reaction mixtures were immunoadsorbed with anti-His-tag antibody resin and analyzed as described under Experimental Procedures. (His_7)-[^{35}S]HRI and coadsorbed FLAG-[^{35}S]PP5, and coadsorbed Hsp90 and p50^{cdc37}, were visualized by autoradiography and Western blotting, respectively. The amount of each protein was quantified by scanning densitometry and expressed as OD*mm^2 (numbers above each panel).

in control reactions (Figure 6A, lane 2). This inhibition was dose-dependent: increasingly higher levels of arachidonic acid led to increasingly greater degrees of HRI inhibition. Like the effects seen for phosphatase inhibitors (Figure 4), the effects of the phosphatase activator arachidonic acid depended on HRI maturation/activation status: application of arachidonic acid after 45 min of HRI maturation/activation had only minor effects on the activity of HRI (Figure 6A, lanes 9−13). Additionally, the electrophoretic gel shift diagnostic of HRI transformation was inhibited by arachidonic acid, an effect consistent with its effect on kinase activity. Equivalent results were seen with linoleic acid, another polyunsaturated fatty acid activator of PP5 (not shown). In contrast, stearic acid, a saturated fatty acid that has little capacity to stimulate PP5's activity in vitro (*34*, *36*), had little inhibitory effect on HRI's transformation and activation compared to similar concentrations of arachidonic or linoleic acid (Figure 6B). Thus, the impact of these fatty acid compounds was specific to the compound's structures rather than representing a nonspecific property of fatty acids per se.

Polyunsaturated Fatty Acids Disrupt the HRI/Hsp90/p50^{cdc37} Heterocomplex in a Dose-Dependent Fashion. Since polyunsaturated fatty acids inhibited the transformation and activation of HRI, we examined their effect on the composition of Hsp90−kinase heterocomplexes. (His_7)-[^{35}S]HRI and FLAG-[^{35}S]PP5 were synthesized in separate heme-replete reticulocyte lysate, after which they were mixed and various concentrations of fatty acids were added. The reaction mixtures were incubated for an additional 20 min, and the composition of chaperone−HRI complexes was subsequently characterized in coadsorption assays (Figure 7).

Addition of arachidonic or linoleic acid to lysate did not result in disruption of the basal (no client) interaction of p50^{cdc37} with Hsp90 (not shown). However, HRI heterocomplexes isolated from reticulocyte lysate treated with arachidonic or linoleic acid showed compromised association of Hsp90 and p50^{cdc37} with immature/mature-competent HRI molecules (Figure 7A,B). This effect was dose-dependent: increasingly higher levels of arachidonic or linoleic acid caused increasingly greater deficiencies in chaperone association (Figure 7A,B). In contrast, arachidonic and linoleic acid treatments had little effect on the interaction of PP5 with HRI. We noted, however, that high concentrations of both arachidonic and linoleic acid resulted in reproducible decreases in levels of (His_7)-[^{35}S]HRI recovered from rabbit reticulocyte lysate. To quantify chaperone losses in a manner that was compensatory for this loss of HRI, protein levels were quantified by densitometry, and the amounts of (His_7)-[^{35}S]HRI, Hsp90, p50^{cdc37}, and PP5 are presented in Figure 7. The data indicate that the relative levels of Hsp90 and p50^{cdc37}, but not PP5, associated with HRI were compromised and declined in concert in arachidonic or linoleic acid treated reticulocyte lysate. Finally, control experiments using stearic

acid confirmed the specificity of the effect: this saturated fatty acid did not disrupt the association of Hsp90 and p50^{cdc37} with HRI (Figure 7C).

DISCUSSION

Work done in numerous laboratories over the past 2 decades has firmly established that phosphorylation plays an essential role in regulating the function of HRI. Nevertheless, the question of whether the function of HRI might be regulated by reversible phosphorylation has remained unclear. In this report, we present for the first time biochemical and pharmacological evidence that the Hsp90-dependent transformation and activation of HRI in response to heme-deficiency are regulated by the activity of a phosphatase, and the data indicate that the phosphatase is likely PP5.

In support of this hypothesis, we find that PP5 is an Hsp90 cohort that occurs in complexes with the Hsp90-dependent kinase HRI. The interaction of PP5 with HRI was dependent upon Hsp90, as mutations in PP5 that inhibit its ability to bind Hsp90 (*28*, *31*) also block the interaction of PP5 with HRI (Figure 3). This effect suggests that Hsp90 plays a direct role in recruiting PP5 to HRI heterocomplexes and would be consistent with PP5's direct association with Hsp90 in the absence of the Hsp90 client.

The interaction of PP5 with HRI occurs in a manner that is distinct from that described previously for other Hsp90 cochaperones (*8*, *13*), as the binding of PP5 molecules to HRI is only partially (50%) inhibited by geldanamycin. The observation that PP5 coadsorbs with HRI in the presence of geldanamycin, despite the quantitative high-salt washing of Hsp90 from HRI/PP5 complexes, indicates that PP5 can interact directly with HRI. However, the ability of mutations that inhibit the interaction of PP5 with Hsp90 to block the binding of PP5 to HRI indicates that the binding of PP5 to HRI is modulated by Hsp90. Together, these results suggest that the interaction of Hsp90 with PP5 is required for the HRI binding site of PP5 to become accessible and that the binding of PP5 to HRI can be initiated through the weak salt-labile interaction that occurs between Hsp90 and HRI in the presence of geldanamycin. However, it is clear that the interaction of PP5 with HRI maturation intermediates differs from that of the Hsp90-regulated cohorts p50^{cdc37} and p23: unlike the binding of p50^{cdc37} and p23 to Hsp90-dependent kinases (*8*, *13*), the binding of PP5 to HRI is not quantitatively dependent upon Hsp90's nucleotide-dependent conformational switching.

Data presented in this paper indicate that PP5 and p50^{cdc37} can occur on the same Hsp90 chaperone machinery and that they can coexist in chaperone−client heterocomplexes. This conclusion derives from the observation that these Hsp90 cohorts co-immunoadsorb under various experimental conditions (Figure 2). Furthermore, expression of the Hsp90−client HRI enhances the association of p50^{cdc37} with PP5 immunocomplexes (Figure 2). The description here of concomitant binding of PP5 and p50^{cdc37} to Hsp90 and Hsp90−client heterocomplexes is consistent with our recent demonstration that p50^{cdc37} and the immunophilin FKBP52 form a novel four-component chaperone machine (*13*). However, the coincident occurrence of p50^{cdc37} on Hsp90 complexes with the TPR-containing cohorts FKBP52 and PP5 is inconsistent with studies that suggest that these components compete for adjacent docking sites on Hsp90 (*18*, *21*, *22*). As we have discussed previously for the four-component Hsp90−p50^{cdc37}−FKBP52−p23 machine (*13*), the discrepancy between our findings and in vitro competition assays may reflect the existence of a dimeric Hsp90 machine (*50*−*54*) in which each Hsp90 subunit binds a different Hsp90 cohort.

Data presented here indicate that one (or more) protein phosphatase(s) has (have) the potential to act upon HRI's Hsp90-dependent activation process. This conclusion derives from the finding that application of the phosphatase inhibitors okadaic acid and nodularin to HRI maturation/activation reactions leads to the generation of *hyperactive* HRI populations (Figure 4). Consistent with this finding, application of compounds that activate PP5 in vitro (arachidonic and linoleic acid) leads to the generation of *hypoactive* HRI populations (Figure 6A). The identities of these agents and their effective concentrations are consistent with the pharmacological profile of PP5 (*34*, *36*, *46*). While the concentration of free arachidonate has been reported to reach 180 μM during brain ischemia (*55*), we feel that it would be premature to propose that the supraphysiological concentrations of unsaturated fatty acids used in this study act similarly in vivo: instead, we have employed these agents simply to manipulate PP5 activity in reticulocyte lysate-based assays. This approach overcomes many of the limitations inherent to the study of PP5, notably, PP5's autoinhibition (*34*, *36*, *56*) and the potential requisite roles of trans-acting PP5 modulators, partners, or competitors. However, we do note that addition of the recombinant PP5 catalytic domain to HRI transformation reactions has effects qualitatively similar to those of fatty acid stimulators: resultant HRI populations have decreased kinase activity.[2]

The phosphatase(s) responsible for these effects can only impact HRI function concomitant with HRI maturation/transformation processes: the pharmacological response of HRI to phosphatase inhibition/activation is wholly dependent upon the timing drug application (Figures 4 and 6). Thus, we propose two models for the action of PP5 on HRI maturation.

In the first model, PP5 may dephosphorylate HRI during its maturation/activation. This interpretation is supported by the finding that HRI with retarded electrophoretic mobility can be detected in Hsp90 heterocomplexes when the phophatase inhibitor NaF was present during the immunoadsorption protocol. Furthermore, this interpretation is consistent with our recent demonstration that phosphorylation of Hsp90-dependent kinases is one possible mechanism for generating Hsp90-independent kinase populations. For the Src-family kinases, Lck and Hck, point mutations of their C-terminal regulatory tyrosines generate kinase populations which show continuous requirements for Hsp90 function (*57*−*59*); however, their wild-type counterparts do not show such a continuous requirement and, instead, flow vectorially from Hsp90-dependent to Hsp90-independent forms. Similarly, HRI is released from its continuous requirement for Hsp90 function by conditions that activate its kinase activity, and release correlates with kinase autophosphorylation (*4*, *8*). Furthermore, Hsp90 function is required for activation

[2] J. Shao and R. L. Matts, unpublished observations.

and phosphorylation of Mos, but once activated, Mos no longer requires Hsp90 to support its function (*60*). However, while circumstantial evidence suggests that kinase phosphorylation may represent an important determinant of kinase maturity and Hsp90 independence, direct correlations between Hsp90 dependence and specific kinase phosphorylation sites have not yet been documented. Nonetheless, these evolving models describing Hsp90's support of protein structure suggest that phosphorylation is an important determinant of Hsp90 dependence, and the physical and functional involvement of PP5 in these processes is consistent with these models. In this model of HRI dephosphorylation coincident with the maturation/activation process, PP5 might modulate HRI's response to activating stimuli. Such modulation could be postulated to be an important tuning of translational repression given HRI's action on eIF2α and the potency of translational inhibition by this mechanism.

As an alternative model, PP5 may act directly upon Hsp90−chaperone machinery to regulate its function(s). While Hsp90 is often described as the "signal-transduction chaperone", due to its apparent specificity for signal transduction proteins, and the Hsp90 chaperone machinery is often described as "regulating" the function of its clients, few characterizations have attempted to discriminate between housekeeping roles for Hsp90 versus regulated roles for Hsp90 in signal transduction. Regarding this last possibility, our data could result from the direct action of PP5 on Hsp90 and/or its partner cochaperones. Consistent with this model, Hsp90 (*61*−*66*), p50^{cdc37} (*67*), p60/HOP (*68*, *69*), and FKBP52 (*70*) are phosphoproteins, and recent studies suggest that the phosphorylation status of Hsp90 machinery may be linked to its chaperoning function (*68*−*75*). Thus, PP5 might regulate Hsp90 function by modulating the phosphorylation status of one or more components of the Hsp90 machine. Models postulating regulated Hsp90 function are further supported by the apparent specificity of cohorts for individual Hsp90 clients (*76*, *77*). This evidence for modulation of Hsp90 function is consistent with the finding that PP5 overexpression negatively regulates glucocorticoid-mediated growth arrest in vivo (*40*) and our observation that PP5 plays a negative role in HRI's Hsp90-dependent maturation/activation.

This negative role for PP5 in HRI's maturation/activation contrasts sharply with the previously described positive role that another Hsp90 partner protein p50^{cdc37} plays during this process (*8*). Thus, PP5 and p50^{cdc37} have opposing influences upon the Hsp90-dependent process of HRI maturation and activation, yet both can coexist in Hsp90−client kinase complexes. Within the context of regulated Hsp90 function, it is reasonable to speculate that these opposing effects must somehow be coordinated during proper execution of Hsp90 function in vivo. Furthermore, it is likely that coordination of the opposing effects of these two proteins plays a role in the regulation of the activity of other Hsp90-dependent signal transduction proteins in addition to HRI.

Irrespective of the finer details by which PP5 may act, our data indicate that this Hsp90 partner protein plays a negative role in regulating the activity of an Hsp90 client during its Hsp90-dependent maturational process. The finding that PP5 downregulates an Hsp90-dependent process supports models for regulated Hsp90 function and describes a novel potential substrate for PP5 function in vivo.

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