Recent studies indicate that p50<sub>cdc37</sub> facilitates Hsp90-mediated biogenesis of certain protein kinases. In this report, we examined whether p50<sub>cdc37</sub> is required for the biogenesis of the heme-regulated eIF2α kinase (HRI) in reticulocyte lysate. p50<sub>cdc37</sub> interacted with nascent HRI co-translationally and this interaction persisted during the maturation and activation of HRI. p50<sub>cdc37</sub> stimulated HRI’s activation in response to heme deficiency, but did not activate HRI per se. p50<sub>cdc37</sub> function was specific to immature and inactive forms of the kinase. Analysis of mutant Cdc37 gene products indicated that the N-terminal portion of p50<sub>cdc37</sub> interacted with immature HRI, but not with Hsp90, while the C-terminal portion of p50<sub>cdc37</sub> interacted with Hsp90. The Hsp90-specific inhibitor geldanamycin disrupted the ability of both Hsp90 and p50<sub>cdc37</sub> to bind HRI and promote its activation, but did not disrupt the native association of p50<sub>cdc37</sub> with Hsp90. A C-terminal truncated mutant of p50<sub>cdc37</sub> inhibited HRI’s activation, prevented the interaction of Hsp90 with HRI, and bound to HRI irrespective of geldanamycin treatment. Additionally, native complexes of HRI with p50<sub>cdc37</sub> were detected in cultured K562 erythroleukemia cells. These results suggest that p50<sub>cdc37</sub> provides an activity essential to HRI biogenesis via a process regulated by nucleotide-mediated conformational switching of its partner Hsp90.

The heme-regulated inhibitor (HRI)<sup>*</sup> of protein synthesis is a protein-serine kinase which coordinates the synthesis of globin chains with the availability of heme in reticulocytes (reviewed in Refs. 1 and 2). Under heme-deficient conditions, HRI phosphorylates the α-subunit of eukaryotic translational initiation factor eIF2. Phosphorylation of eIF2α causes an inhibition of polypeptide chain initiation and the arrest of protein synthesis, preventing the synthesis of apo-globin chains in the absence of heme. HRI is also activated under heme-replete conditions in response to a host of other adverse environmental stimuli, such as heat shock, agents that generate oxidative stress, and the presence of denatured proteins (1, 2).

The biogenesis and activation of HRI into an active heme-regulatable eIF2α kinase requires its functional interaction with the chaperone machinery containing the 90-kDa heat shock protein (Hsp90) and the 70-kDa heat shock cognate protein (Hsc70) (3, 4). During HRI biogenesis and its subsequent transformation and activation, several discrete HRI intermediates are generated; these intermediates can be distinguished on the basis of their competence to become an active kinase in response to heme deficiency or upon treatment with sulphydryl reactive compounds such as N-ethylmaleimide. Immediately after their synthesis, HRI molecules are not active in heme-replete or heme-deficient rabbit reticulocyte lysate (RRL) and cannot be activated by N-ethylmaleimide treatment. This immature population interacts with Hsp90 and Hsc70 (3–7). Subsequent to this immature phase, a “mature-competent” HRI population appears. The mature-competent population can be activated by heme-deficiency or treatment with N-ethylmaleimide, but remains quiescent in the absence of such “stimuli.” The mature-competent HRI population continues to interact with Hsp90 machinery, and this interaction is required to maintain HRI’s ability to respond to heme deficiency (3).

Under heme-deficient conditions, however, a portion of the population of HRI molecules “transform” to produce kinase populations with enhanced auto-kinase and eIF2α kinase activities. Transformation of HRI requires Hsp90 function and autophosphorylation of HRI, and correlates with the production of a population of HRI molecules which exhibit retarded electrophoretic mobility on SDS-PAGE (3). This transformation frees HRI from its functional requirements for Hsp90 and terminates its physical association with Hsp90 machinery (3).

Hsp90 binds numerous other protein kinases, primarily when they are in comparatively inactive conformations (reviewed in Refs. 8 and 9). However, Hsp90’s association with inactive kinases reflects its essential positive role in facilitating kinase folding, maturation, and activation rather than a recognition of repressed kinase molecules per se. Consistent with this model for Hsp90 function, the Hsp90 chaperone machine does not interact with previously transformed HRI molecules when their kinase activity is subsequently inhibited by hemin addition, nor do such “repressed” HRI molecules require Hsp90 support to maintain their ability to reactivate in response to heme-deficiency or stress (3). Consistent with the specific role
of Hsp90 in kinase biogenesis, both repressed HRI and transformed HRI exhibit the same slow electrophoretic mobility on SDS-PAGE, indicating that repressed HRI retains the hsp90-independent "transformed" conformation (3).

Hsp90 functions in concert with a number of co-chaperones and cofactors to facilitate their clients’ acquisition of functional conformations (reviewed in Ref. 10). Early studies detected a 50-kDa protein present in heterocomplexes formed between Hsp90 and viral members of the Src family of protein tyrosine kinases (reviewed in Ref. 11). This 50-kDa protein has recently been identified as a product of the vertebrate homolog of the yeast cell division cycle gene CDC37 (12–16). In vitro, the yeast p50cdc37 homolog exhibits chaperone activity (17). Genetic (17–20) and biochemical (14, 21) studies indicate that p50cdc37 plays an essential positive role in supporting a number of protein kinases. Therefore, we examined whether p50cdc37 is a component of the Hsp90 chaperone complex which is required for the maturation and transformation of HRI. In this report, we present evidence that p50cdc37 acts in concert with Hsp90 and facilitates the transformation and activation of HRI. We further demonstrate that nucleotide-mediated conformational switching of Hsp90 regulates the kinase binding activity of p50cdc37.

EXPERIMENTAL PROCEDURES

Construction of Plasmids for Expression of Wild Type and Mutant p50cdc37 Proteins—Two cDNA clones were obtained from Genome Systems, Inc., one lacking sequences encoding the N-terminal 8 amino acids (MVDSVWD) of human p50cdc37 (dbEST number 810394, GenBank™ accession number AA172101 (designated here as p50cdc37/N8aa)); and another containing sequence encoding the full-length human p50cdc37. These cDNA sequences were cloned into a modified version of pSP64T (22), and a modified version of pQE-32 expression vector (Qiagen). (His6)-p50cdc37 was expressed in M15/pREP4 cells and purified on Ni2+-NTA resin (Qiagen). Coomassie Blue staining of the purified proteins separated by SDS-PAGE indicated that the proteins were greater than 90% pure.

For in vitro translation, sequences encoding p50cdc37/N8aa or full-length p50cdc37 were cloned into a modified version of pSP64T (22), and translated by coupled transcription/translation in RRL (23). The mutan t p50cdc37/N8aa protein produced includes 11 N-terminal amino acids (MVDVSVDV) encoded by sequence carried over from the pet-30a(+) vector, followed by the coding sequence of the human Cdc37 gene product from Has to Val378. The previously described N-terminal deletion mutant of human p50cdc37 lacking 163 amino acids from its N terminus (21) (designated here as p50cdc37ΔN) was similarly cloned into and expressed from pSP64T. Tryptic fingerprint spectra were generated from wild type (53Sp/p50cdc37) and (53Sp/p50cdc37/N8aa) proteins, as described previously for de novo synthesized (53Sp/p50cdc37) (21).

Expression and Purification of p50cdc37 Protein Constructs from Eukaryotic Cells—Human FLAG-tagged p50cdc37 and FLAG-tagged p50cdc37ΔC were expressed in and purified from SF9 cell as described previously (21). FLAG-p50cdc37ΔC lacks 214 amino acids from the C terminus of p50cdc37. GST-tagged p50cdc37 was also expressed in and purified from COS-1 cells as described previously (21).

Analysis of the Association of p50cdc37 with HRI in Cultured Mammalian Cells—Human K562 erythroleukemia cells were cultured for 48 h at 37 °C and 5% CO2 in RPMI medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Approximately 1 × 107 geldanamycin-treated (1 μM for 2 h) or untreated (equivalent volume of MeSO for 2 h) cells were lysed in lysis buffer containing 10 mm HEPES, pH 7.5, 100 mm NaCl, 0.1% Nonidet P-40, 1% Triton X-100, 100 μg/mL glycerol, 1 μM Na3VO4, 2 μM EGTa, 1 μM dithiothreitol, 50 mm glyceraldehyde-3-phosphate, 1 mm NaF, 0.4 mm phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin and aprotinin, and cell extracts were clarified. Endogenous p50cdc37 was immunoadsorbed from cell extracts via a mixture of rabbit, and mouse anti-p50cdc37 (Transduction Laboratories) antibodies bound to GammaBind-Plus Sepharose (Amersham Pharma cia Biotech), and the immunopellets were washed as described previously (21). Half of the recovered p50cdc37 immunocomplexes were analyzed by Western/ECL (Amersham Pharmacia Biotech) for the presence of associated HRI, using guinea pig antibody raised against the N-terminal 154 amino acids of rabbit HRI. Blots were then stripped, and rabbit anti-p50cdc37 antibody was used to verify that equivalent amounts of p50cdc37 were adsorbed from each extract. The second half of the anti-p50cdc37 immunocomplexes were analyzed by Western blotting with rat anti-Hsp90 monoclonal antibody (SPA-830 from Stressgen).

De Novo Synthesis and Maturation of HRI in Rabbit Retinalocyte Lysate—Coupled transcription/translation (TnT) of plasmids encoding HRI or (His6)-HRI in rabbit retinalocyte lysate (TnT RRL) and the subsequent maturation of the expressed HRI or (His6)-HRI in hemin-supplemented or heme-deficient RRL was carried out as described previously (3, 4).

Co-translational Interaction of Hsp90 and p50cdc37 with HRI—HRI and luciferase were synthesized with concomitant 35S-radiolabeling in separate TnT RRL for 15 min at 30 °C. TnT RRL was either treated, or not treated with 1 μM puromycin for 5 min at 30 °C to release the nascent polypeptide chains from polyribosome. The polyribosomes were then isolated by centrifugation and analyzed by Western blotting for Hsp90 and p50cdc37 as described previously (3, 4).

Immunoadsorption of Protein Complexes—Chaperone/cochaperone and chaperone/kinase heterocomplexes were analyzed by reciprocal immunoadsorptions utilizing resin-bound anti-p50cdc37 and anti-Hi stagged antibodies as described previously (23). Kemptide (20 μg/mL) (MOPC 21 from Sigma) was used as a control for nonspecific binding. As an additional control for nonspecific binding, [35S]HRI lacking the histidine tag was assayed in parallel with reactions containing (His6)-[35S]HRI. Relative amounts of immunoadsorbed proteins were quantified by scanning densitometry of autoradiograms or Western blots. Comparisons of changes in the relative protein levels made in the text reflect corrections made for levels of nonspecific binding.

Assay of the Effect of p50cdc37 and p50cdc37ΔC on HRI Transformation—[His6]-HRI or (His6)-[35S]HRI was synthesized in TnT RRL (3, 4). Subsequently, 4 μl of the TnT RRL was transferred to heme-deficient RRL (30 μl) which contained 10 μg of immunorein (M2 anti-FLAG-agarose) that had been previously saturated with FLAG-peptide, FLAG-agarose, or FLAG-agarose buffer. The reaction mixtures were incubated at 30 °C for 1 h. After washing of the immunoreins, the immunoreins were analyzed for bound [35S]HRI, HRI kinase activity, and/or associated chaperones, as specified in the figure legends.

Alternatively, [35S]HRI or (His6)-[35S]HRI were chased into 7 volumes of heme-deficient protein synthesizing RRL containing purified (His6)-p50cdc37 (1.5 μg/μl), GST-p50cdc37ΔC (1 μg/μl), or an equivalent volume of the appropriate control buffer. After 1 h of maturation at 30 °C, samples were adsorbed to Ni2+-NTA resin (Fig. 6B) or GaG-agarose containing bound mouse monoclonal anti-(His6)-IgG (Fig. 7). Resins were subsequently washed and analyzed for kinase activity (3, 4).

Assay of the Kinase Activity of (His6)-[35S]HRI—To quantify HRI kinase activity, (His6)-[35S]HRI or (His6)-[35S]HRI (control for nonspecific binding) was captured from RRL reaction mixtures on Ni2+-NTA-agarose (Qiagen) that had been pre-equilibrated with 10 mM Tris-HCl, pH 7.4, 10 mM imidazole, and 50 mM NaF, or by immunoadsorption with anti-(His6)-monoclonal antibody. Assays for eIF2α kinase activity were performed as described (3, 4). The kinase activity of HRI was quantified by scanning densitometry of the 3P-labeled eIF2α band visualized by autoradiography and expressed as optical density (O.D.) × mm2.

RESULTS

p50cdc37 Interacts with HRI in Concert with Hsp90—To determine whether p50cdc37 was a component of the chaperone complex that Hsp90 forms with HRI, we examined the ability of anti-His-tag antibodies to co-adsorb Hsp90 and p50cdc37 upon immunoprecipitation of newly synthesized (His6)-[35S]HRI from RRL (Fig. 1A). As previously shown (3), immunoadsorption of (His6)-[35S]HRI folding intermediates immediately after their synthesis (5 min post-translation) specifically co-adsorbed Hsp90. Immunoadsorption of newly synthesized HRI also co-adsorbed p50cdc37, demonstrating the existence of a heterocomplex containing p50cdc37 and these newly synthesized HRI molecules. The amount of Hsp90 and p50cdc37 co-adsorbed with HRI was similar whether the newly synthesized HRI was immunoadsorbed from heme-replete or heme-deficient RRL.
forms into an active kinase that no longer interacts with Hsp90 (3). To determine whether transformation of HRI similarly terminated its interaction with p50\textsuperscript{cdc37}, the newly synthesized HRI kinase population was incubated in heme-deficient RRL for 60 min. Under these conditions, ~50% of the pulse-labeled (His\textsubscript{4})\textsuperscript{[35S]}HRI exhibited the slower electrophoretic mobility associated with “transformation” (3). The amount of Hsp90 and p50\textsuperscript{cdc37} that was co-adsorbed with (His\textsubscript{4})-HRI from heme-deficient RRL decreased by ~67 and 75%, respectively, relative to that co-adsorbed with the “mature-competent/untransformed” HRI population present in RRL held under heme-replete conditions (Fig. 1A, lane 2 versus 4). Thus, the transformation of HRI that was induced by heme deficiency correlated with a reduction in the interaction of HRI with Hsp90 and p50\textsuperscript{cdc37}.

Previously, the interaction between Hsp90 and HRI was demonstrated to be specific to the “untransformed” (fast electrophoretic mobility) form of HRI (3). To determine whether p50\textsuperscript{cdc37} similarly binds to HRI during its synthesis on ribosomes, polypeptides were isolated from RRL that was programed with HRI template. For a negative control, ribosomes were also prepared from RRL that was programmed with luciferase template. Western blotting indicated that both Hsp90 and p50\textsuperscript{cdc37} were present in the ribosome pellet containing bound nascent HRI polypeptide chains (Fig. 1B), but were absent when the nascent polypeptide chains had been released by incubation with puromycin prior to isolation of the polyribosomes. Thus, p50\textsuperscript{cdc37}, like Hsp90, becomes specifically associated with HRI prior to the completion of kinase synthesis and the release of newly synthesized HRI from the ribosome.

The interaction of Hsp90 with HRI persists after release of newly synthesized HRI from polyribosomes in heme-replete RRL (3). To assess if the interaction between p50\textsuperscript{cdc37} and HRI was similarly maintained, the newly synthesized (His\textsubscript{4})\textsuperscript{[35S]}HRI population was subjected to maturation incubations in heme-supplemented RRL, where the transformation of HRI into an active kinase is suppressed. After a 60-min incubation in the presence of hemin, neither the level of Hsp90 nor p50\textsuperscript{cdc37} associated with HRI declined significantly relative to the level observed immediately after translation (Fig. 1A, lane 4 versus 6 and 8). Thus, in heme-replete RRL, HRI continued to interact with p50\textsuperscript{cdc37} for prolonged periods after its synthesis.

In heme-deficient RRL, HRI autoposphorylates and trans-
The above result implied that the C-terminal truncated Cdc37 gene products could bind kinase folding intermediates independent of Hsp90 function. To test this, the effects of poisoning RRL with the Hsp90-specific antagonist geldanamycin (25, 26) were assessed. Consistent with previous reports (14, 21), the ability of full-length FLAG-p50cdc37 gene product to bind untransformed kinase molecules was markedly reduced (67%) in the presence of geldanamycin (Fig. 3A, upper panel), but the presence of geldanamycin had no inhibitory effect on the interaction of FLAG-p50cdc37 with Hsp90 (Fig. 3A, middle panel). In contrast, inhibition of Hsp90 function by geldanamycin did not abrogate the ability of the truncated Cdc37 gene product p50cdc37ΔC to bind untransformed HRI. In fact, geldanamycin treatment appeared to potentiate the kinase binding activity of truncated Cdc37 gene products. Thus, the resistance of the interaction between p50cdc37ΔC and HRI to inhibition by geldanamycin indicated that the N-terminal half of the Cdc37 gene product was capable of binding kinase folding intermediates independent of Hsp90.

The effects of geldanamycin on chaperone binding to untransformed HRI intermediates was confirmed by the reciprocal co-adsorption assays (Fig. 3B). (His7)-HRI was immunoadsorbed from control or geldanamycin-treated RRL and analyzed for the presence of co-adsorbed Hsp90 and p50cdc37. While the association of Hsp90 and p50cdc37 with (His7)-HRI in control RRL was readily detected, treatment of RRL with geldanamycin resulted in the near quantitative disruption of the association of Hsp90 and p50cdc37 with (His7)-HRI (Fig. 3B), thus confirming that inhibition of Hsp90 disrupted the direct recognition of kinase molecules by p50cdc37.

The failure of C-terminal deleted p50cdc37 to bind Hsp90 (21) suggested that the elements of p50cdc37 which mediated its interaction with Hsp90 resided in the C-terminal domain of p50cdc37. To further differentiate whether the C-terminal region of p50cdc37 contained the Hsp90-binding site or whether it was simply required to maintain the structure of an Hsp90-binding site elsewhere in the protein, we constructed the corresponding N-terminal deletion mutant of p50cdc37 lacking the first 163 amino acids from its N terminus (p50cdc37ΔN). p50cdc37ΔN was synthesized in TnT RRL and assayed for Hsp90 binding. Anti-Hsp90 antibody specifically co-adsorbed [35S]p50cdc37ΔN in conjunction with Hsp90 (Fig. 4A). Furthermore, anti-His antibodies specifically co-adsorbed Hsp90 with His-tagged p50cdc37ΔN (Fig. 4B). These findings indicated that the C-terminal domain of p50cdc37 contained elements adequate for Hsp90 binding, independent of the presence of p50cdc37ΔN-terminal domain.

**Mutation of the N Terminus of p50cdc37 Inhibits Its Binding to HRI**—To further characterize the domains and motifs of p50cdc37 required for kinase binding, we utilized a p50cdc37 mutant whose first 8 N-terminal amino acids were replaced by 11 residues encoded by irrelevant plasmid sequence (p50cdc37/N8aa). Two observations indicated that replacement of the first 8 residues of p50cdc37 did not cause global disruption of p50cdc37 structure. (i) This mutation did not compromise the ability of [35S]p50cdc37/N8aa to associate with Hsp90 (Fig. 5A). (ii) This mutation did not compromise the structural integrity of p50cdc37/N8aa as determined by mild proteolytic knicking: the sensitivity to protease digestion and the pattern of proteolytic fragments generated were essentially the same for the p50cdc37/N8aa and the wild type p50cdc37 proteins (Fig. 5B). However, p50cdc37/N8aa was not co-adsorbed with epitope-tagged (His7)-HRI despite the efficient co-adsorption of HRI by wild-type p50cdc37 and the equivalent amounts of each Cdc37 gene product present in these assays (Fig. 5C). These observations demonstrated that the N-terminal 8 amino acids of p50cdc37 were

Previously, we have demonstrated that the kinase activity of transformed HRI is inhibited upon addition of hemin to heme-deficient maturation mixtures, and that this population of “repressed HRI” molecules does not interact with Hsp90 (3). Similarly, we have observed that repressed HRI did not co-adsorb with p50cdc37 from RRL (not shown). Thus, p50cdc37 did not bind to transformed HRI regardless of whether the kinase was active or repressed.

**The N-terminal Domain of p50cdc37 Binds HRI Independent of Hsp90**—Hsp90 and p50cdc37 both recognize untransformed populations of HRI molecules (Fig. 1 and 2). To differentiate between p50cdc37/ associate with HRI directly or binding HRI indirectly via its interaction with Hsp90, we compared the kinase binding activity of affinity-purified TAG-p50cdc37 with that of a C-terminal truncated FLAG-tagged Cdc37 gene product (p50cdc37ΔC). p50cdc37ΔC had previously been shown to bind the Raf-1 kinase, but not Hsp90 (21). HRI was readily captured by resins containing the full-length wild-type Cdc37 gene product and by resins containing the truncated Cdc37 gene product p50cdc37ΔC, but was not captured by anti-FLAG control resins (Fig. 3A, upper panel). In contrast, Hsp90 was adsorbed by resins containing the wild-type Cdc37 gene product, but only barely detectable amounts of Hsp90 were bound to resins containing p50cdc37ΔC truncated Cdc37 gene product (Fig. 3A, middle panel). These findings suggested that Cdc37 gene products did not bind to kinase folding intermediates indirectly via an interaction with kinase-bound Hsp90.
essential for its interaction with HRI.

*p50<sup>cdc37</sup> Enhances HRI Activity in Heme-deficient RRL in an Hsp90-dependent Fashion—To further characterize the role of p50<sup>cdc37</sup> in the biogenesis of HRI, we examined the effect of affinity purified (His<sub>6</sub>)-p50<sup>cdc37</sup> on the transformation and activation of newly synthesized [35S]HRI in heme-deficient RRL (Fig. 6). Aliquots of the HRI transformation reactions were also taken at the indicated times and analyzed by SDS-PAGE for transformation-specific shifts in HRI's electrophoretic mobility (Fig. 6A). Maturation of HRI in heme-deficient RRL containing (His<sub>6</sub>)-p50<sup>cdc37</sup> produced transformed HRI molecules equivalent to the storage buffer control. However, inclusion of (His<sub>6</sub>)-p50<sup>cdc37</sup> in HRI activation reactions increased the proportion of transformed HRI that became hyperphosphorylated at all time points examined relative to the control. Results similar to those presented in Fig. 6 were also obtained upon supplementing RRL with GST-tagged p50<sup>cdc37</sup> (not shown: e.g., see Fig. 7).

The effect of p50<sup>cdc37</sup> on the eIF2α kinase activity of HRI was also assessed (Fig. 6B). After 60 min of incubation, the eIF2α kinase activity of HRI matured in p50<sup>cdc37</sup>-supplemented heme-deficient RRL was nearly 3-fold higher relative to HRI matured in control RRL supplemented with buffer alone. Thus, the physical association of p50<sup>cdc37</sup> with untransformed intermediates of HRI appeared to reflect a positive role for this co-chaperone in HRI maturation/activation pathway, as supplementation of RRL with p50<sup>cdc37</sup> promoted the acquisition of an active conformation.

The data presented in Fig. 3 indicated that p50<sup>cdc37</sup> required geldanamycin-inhibitable Hsp90 function for it to form a stable complex with untransformed HRI intermediates. To determine whether endogenous Hsp90 function was also required for the stimulatory effects of recombinant p50<sup>cdc37</sup> on HRI transformation and activity, the effect of including geldanamycin in the transformation-activation assays was assessed (Fig. 7). Similar to the results obtained with (His<sub>6</sub>)-p50<sup>cdc37</sup>, supplementation of RRL with recombinant GST-p50<sup>cdc37</sup> enhanced the eIF2α kinase activity of HRI 3-fold over the control, and this enhancement of activity correlated with the generation of a [35S]HRI species with a slower electrophoretic mobility (lower panel, denoted HP). Addition of geldanamycin inhibited the activation of HRI in both buffer-supplemented control and p50<sup>cdc37</sup>-supplemented heme-deficient RRL (Fig. 7, upper panel). Consistent with this finding, the slow electrophoretic form of HRI representing transformed HRI molecules was not produced in geldanamycin-poisoned reactions (Fig. 7, lower panel). These results indicated that geldanamycin-inhibitable Hsp90 function was essential for the observed stimulatory effects of p50<sup>cdc37</sup> on HRI transformation-activation.

To further test the hypothesis that the positive effect which p50<sup>cdc37</sup> has on HRI activity is modulated through its interaction with Hsp90, we examined the effect of the p50<sup>cdc37</sup>/ΔC mutant on the transformation and activation of HRI in heme-deficient RRL (Fig. 8). The data presented in Fig. 3 indicate that complexes formed between HRI and the p50<sup>cdc37</sup>/ΔC mutant lacked bound Hsp90. Thus, the p50<sup>cdc37</sup>/ΔC mutant was predicted to retard HRI transformation and activation when added to heme-deficient RRL. (His<sub>6</sub>)-HRI (Fig. 8, even lanes) or non-Flag-tagged HRI (Fig. 8, odd lanes) was synthesized and then matured in heme-deficient RRL in the presence of control M2 anti-Flag tag-agarose saturated with FLAG-peptide, FLAG-tagged-p50<sup>cdc37</sup>, or FLAG-tagged-p50<sup>cdc37</sup>/ΔC. The electrophoretic mobility of the non-Flag-tagged [35S]HRI that was specifically associated with p50<sup>cdc37</sup> or p50<sup>cdc37</sup>/ΔC indicated that the HRI was not transformed, and the p50<sup>cdc37</sup>/ΔC-bound non-Flag-tagged [35S]HRI were as inactive in phosphorylating eIF2α as the material present in the control pellet for nonspecifically bound activity (Fig. 8, lanes 1, 3, and 5).

The effects of the p50<sup>cdc37</sup> and p50<sup>cdc37</sup>/ΔC mutant on the eIF2α kinase activity and electrophoretic properties of the total (His<sub>6</sub>)-[35S]HRI population matured in these RRLs and subsequently adsorbed to anti-Hsp-resin were also examined after adsorption (Fig. 8, lanes 2, 4, and 6). The eIF2α kinase activity of the (His<sub>6</sub>)-[35S]HRI that was matured in heme-deficient RRL in the presence of wild type p50<sup>cdc37</sup> was almost twice as much as the eIF2α kinase activity present in the control RRL con-
containing M2 anti-FLAG tag-agarose with bound FLAG-peptide. In contrast, the elF2α kinase activity of the (His7)-HRI that was matured in the presence of the p50cdc37ΔMC mutant was decreased by 60% relative to the kinase activity adsorbed from control RRL. A portion of the (His7)-[35S]HRI that was matured in the presence of wild type p50cdc37 exhibited a slower electrophoretic mobility than the (His7)-[35S]HRI that was matured in control RRL, indicating it had become hyperphosphorylated (Fig. 8, lower panel, lane 4 versus 2 HP)). This shift in electrophoretic mobility caused the band to appear more diffuse. However, quantification of the total amount of (His7)-[35S]HRI molecules exhibiting slower electrophoretic mobilities indicated that nearly the same amount of (His7)-[35S]HRI became transferred in RRL supplemented with wild type p50cdc37 (O.D. × mm² = 4.14) compared with control RRL (O.D. × mm² = 4.11). This observation is consistent with the enhanced elF2α kinase activity of adsorbed HRI was assayed as described under “Experimental Procedures.” Samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and visualized by autoradiography. The amount of [32P]eIF2α was quantified by scanning densitometry and expressed as optical density × mm² (numbers above the lanes in panel). B, for analysis of HRI activity, aliquots (50 μl) of the RRL reaction mixtures containing His7-HRI, His7-HRI + cdc37TR (lanes 1 and 5) or His7-HRI + cdc37MC (lanes 2 and 6) were taken after 60 min of incubation at 30 °C and adsorbed to Ni²⁺-NTA-agarose as described under “Experimental Procedures.” After washing away unbound materials, the elF2α kinase activity of the adsorbed HRI was assayed as described under “Experimental Procedures.” Samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and [32P]eIF2α (B, lower panel) were detected by autoradiography. The amount of [32P]eIF2α was quantified by scanning densitometry and expressed as optical density × mm² (numbers above the elF2α panel). Densitometry indicated that equivalent amounts of total [35S]HRI were specifically immunoadsorbed (lanes 3 and 4). HP, hyperphosphorylated form of HRI; TR, transformed form of HRI; MC, mature competent form of HRI.
activity displayed by the (His<sub>7</sub>)-[35S]HRI that was matured in the presence of wild type p50<sup>cdc37</sup>. In addition, the fast electrophoretic mobility of the (His<sub>7</sub>)-[35S]HRI that was matured in the presence of p50<sup>cdc37</sup>/ΔC indicated that it remained mostly untransformed, consistent with its suppressed kinase activity (Fig. 8, lane 6). These results indicate that Cdc37 gene products which do not interact with Hsp90 may act as dominant negative inhibitors of p50<sup>cdc37</sup> function: a conclusion consistent with the observation that Hsp90 is required for p50<sup>cdc37</sup> to exert its positive effect on HRI activity upon transformation.

p50<sup>cdc37</sup> Does Not Enhance HRI Activity after Its Transformation—HRI can become further activated in RRL after its transformation and release from Hsp90. This further activation of transformed HRI is negatively attenuated by the interaction of Hac70 with HRI, and is also accompanied by HRI's hyperphosphorylation (4). To verify that p50<sup>cdc37</sup> functions are specific to untransformed HRI, HRI was transformed in hemin-deficient RRL prior to the addition of recombinant p50<sup>cdc37</sup> (Fig. 9). As an additional control, denatured (reduced carboxymethylated) bovine serum albumin was similarly added to heme-deficient RRL after the completion of HRI's transformation to block the ability of Hac70 to negatively attenuate HRI's activation (4, 27, 28). Addition of reduced carboxymethylated bovine serum albumin to heme-deficient RRL stimulated the eIF2α kinase activity of HRI 4-fold compared with the activity observed in control RRL. In contrast, the addition of an equivalent amount of recombinant p50<sup>cdc37</sup> to heme-deficient RRL subsequent to HRI transformation caused little further stimulation of HRI's eIF2α kinase activity (approximately 10%) compared with the control. Therefore, the stimulatory effects of p50<sup>cdc37</sup> were specific to that HRI population whose transformation had been previously documented to be functionally dependent upon geldanamycin-inhibitable Hsp90 function.

Interaction of p50<sup>cdc37</sup> with HRI in Vivo—Interactions between endogenously expressed p50<sup>cdc37</sup> and HRI were examined in the chronic myelogenous leukemic K562 cell line to determine whether p50<sup>cdc37</sup> also interacted with HRI in vivo. K562 cells, cultured in the presence or absence of geldanamycin for 2 h, were lysed and endogenous p50<sup>cdc37</sup> was immunoadsorbed (Fig. 10). Immunoblotting with HRI-specific antibodies indicated that HRI was co-adsorbed with p50<sup>cdc37</sup> from extracts of control cells (Fig. 10, upper panel). However, treatment of K562 cells with geldanamycin abolished the interaction of p50<sup>cdc37</sup> with HRI. In contrast, geldanamycin treatment of K562 cells had no effect on the avid interaction between p50<sup>cdc37</sup> and Hsp90 in vivo (Fig. 10, middle panel). Stripping and reprobing with anti-p50<sup>cdc37</sup> antisera verified that equivalent amounts of p50<sup>cdc37</sup> were immunoprecipitated from the extracts of the untreated and geldanamycin-treated cells (Fig. 10, lower panel). These results indicated that HRI existed in geldanamycin-sensitive native complexes with p50<sup>cdc37</sup> in cultured K562 cells.

**DISCUSSION**

p50<sup>cdc37</sup> plays a positive role in facilitating activation of HRI in response to heme-deficiency. Supplementation of heme-deficient RRL with recombinant p50<sup>cdc37</sup> during the HRI activation process stimulates the production of HRI populations with enhanced kinase activity and stimulates the production of HRI populations with retarded electrophoretic mobilities which are diagnostic of HRI activation/transformation. While our present work is the first to directly address the role of p50<sup>cdc37</sup> in kinase biogenesis in an *in vitro* model system, these observations are consistent with biochemical and genetic data which indicate that p50<sup>cdc37</sup> plays an essential positive role in supporting the function of numerous protein kinases (14, 17, 19–21, 29, 30). This positive function is consistent with a primary role for p50<sup>cdc37</sup> in a striking partnership with Hsp90 as a kinase-specific corepressor.

The stimulatory effect of p50<sup>cdc37</sup> is specific to the HRI activation process: p50<sup>cdc37</sup> has no effect on the kinase activity or electrophoretic mobility of HRI when added subsequent to HRI's transformation. Consistent with this specificity, p50<sup>cdc37</sup> interacts with newly synthesized HRI and with the inactive HRI population maintained in the presence of hemin, but does not interact with HRI that has been transformed in response to heme deficiency or with transformed HRI whose activity has been repressed by hemin. Therefore, like Hsp90's association with inactive kinases, the interaction of p50<sup>cdc37</sup> with HRI
structures present within the N-terminal half of p50\textsubscript{cdc37}. We demonstrate here that the C-terminal half of p50\textsubscript{cdc37} contains semi-autonomous Hsp90 binding activity (Fig. 4). Thus, sequences or structures present in the C-terminal region of p50\textsubscript{cdc37} directly mediate its binding to Hsp90. However, despite the observation that the N-terminal domain of p50\textsubscript{cdc37} does not stably interact with Hsp90, we cannot rule out the possibility that motifs in regions outside of the C terminus of p50\textsubscript{cdc37} may also contribute directly or indirectly to the interaction of p50\textsubscript{cdc37} with Hsp90.

Our data indicate that p50\textsubscript{cdc37} exerts its biochemical effects in cooperation with Hsp90. This conclusion is indicated by the observation that the Hsp90-specific inhibitor geldanamycin inhibits the ability of recombinant p50\textsubscript{cdc37} to stimulate HRI transformation (Fig. 7). Geldanamycin similarly inhibits the ability of p50\textsubscript{cdc37} overexpression to stimulate Raf activation in cultured cells (21), indicating that Hsp90 is required for p50\textsubscript{cdc37} to exert its positive effect on Raf-1 activation \textit{in vivo}. Furthermore, these results do not reflect a direct disruption of the Hsp90-p50\textsubscript{cdc37} interaction (Figs. 3 and 10) (21). Instead, geldanamycin disrupts the normal association of p50\textsubscript{cdc37} with HRI in RRL and in cultured K562 cells (Figs. 3 and 10). This finding is consistent with previous characterization of the effects of geldanamycin on p50\textsubscript{cdc37} interactions with CDK4, Raf-1, and KSR \textit{in vivo} (14, 21, 32).

In contrast, geldanamycin does not inhibit the ability of C-terminal truncated Cdc37 gene products to bind kinase-folding intermediates; inhibition of Hsp90 actually increases the availability of HRI molecules for binding to truncated cdc37 proteins (Fig. 3). This observation has 3 important implications. 1) Geldanamycin’s disruption of p50\textsubscript{cdc37}, kinase heterocomplexes does not result from sequestration or masking of kinase-folding intermediates by geldanamycin-poisoned Hsp90 machinery. 2) The physical interaction of full-length p50\textsubscript{cdc37} with Hsp90 determines geldanamycin’s ability to inhibit the binding of p50\textsubscript{cdc37} to kinase molecules. 3) Geldanamycin-inhibitable Hsp90 function is necessary to assemble heterocomplexes formed directly between kinase molecules and full-length p50\textsubscript{cdc37} and to chaperone the kinase substrate toward its activation-specific conformation.

Geldanamycin is known to act as a specific inhibitor of Hsp90 through its ability to bind avidly within Hsp90’s nucleotide-binding pocket, thus blocking the binding of ATP (26, 33, 34). This binding prevents nucleotide-mediated switching between alternative Hsp90 conformations (33, 35–37) and abolishes Hsp90’s ability to establish high affinity interactions with its substrates (23, 37), thus inhibiting Hsp90-supported kinase function. Therefore, geldanamycin’s ability to inhibit the binding of wild type p50\textsubscript{cdc37} to kinase-folding intermediates demonstrates that Hsp90’s nucleotide-mediated conformational switching regulates the direct binding of p50\textsubscript{cdc37} to Hsp90’s kinase clients. Additionally, our data illuminate potential mechanisms underlying the chemotherapeutic potential of benzozquinonoid ansamycins.

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