Checkpiong Checkpoint Kinase 1 (Chk1), an Hsp90 Client, with Purified Chaperones*

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Hsp90 is a part of a ubiquitously expressed multiprotein molecular chaperone system that is required for the folding, maturation, and stabilization of specific client proteins (reviewed in Refs. 1 and 2). Hsp90 is an integral part of a multiprotein complex that transitions between at least two forms, dependent on whether or not Hsp90 is bound to ATP. In a steroid receptor model system, ATP-bound Hsp90 complexes with the co-chaperones p23 and immunophilins, and this complex promotes client protein stabilization. On the other hand, Hsp90 lacking nucleotide or bound to ADP is associated with Hsp70 and Hop. This intermediate stage complex can also promote proteasomal degradation of client proteins.

Hsp90 inhibitors, such as geldanamycin, radicicol, and their derivatives, disrupt Hsp90 function by mimicking the ADP-bound conformation and targeting client proteins for proteasomal degradation, thereby depleting client proteins from treated cells (reviewed in Refs. 3–5). The discovery of specific Hsp90 inhibitors has facilitated the identification of Hsp90 clients, leading to a vast expansion of the list of Hsp90 clients. Notably, many Hsp90 clients, such as Akt, Raf-1, and Her2/Neu, play important roles in tumor cell survival and proliferation, which has further fueled the interest in the role of Hsp90 in client protein function, especially the role of Hsp90 in regulating protein kinase clients.

The Hsp90 chaperoning process has been studied most extensively for the steroid receptors. These studies have demonstrated that five purified proteins: Hsp90, Hsp70, Hsp40, p23, and Hop, are sufficient to chaperone steroid receptors in vitro into Hsp90-receptor complexes capable of binding hormone (6–9). This process first involves binding of the receptor by Hsp40 and Hsp70 (10, 11). The co-chaperone Hop then recruits Hsp90 to the Hsp70-Hsp40-receptor complex (12). With the binding of ATP to Hsp90, Hsp70, Hsp40, and Hop dissociate from the complex and are replaced by p23 (8). It is this mature complex containing receptor bound to Hsp90 and p23 that has hormone binding activity.

The detailed characterization of the chaperoning process of steroid receptors has provided important insights into the chaperoning process and the roles played by various chaperone proteins. Additional studies have demonstrated that chaperoning of hepatitis B virus reverse transcriptase requires the same five proteins (13, 14). Despite this progress, no in vitro system using purified proteins has been established to study protein kinases, an important class of Hsp90 clients that plays key roles in regulating tumor cell proliferation and survival. This is particularly relevant because a large number of the Hsp90 clients discovered to date are protein kinases, and yet the molecular details of how they are chaperoned remain elusive.

In the present work we demonstrate the first successful fully defined system for in vitro chaperoning of a protein kinase using purified proteins. Our studies show that Chk1, a kinase that was recently identified as an Hsp90 client, can be post-translationally chaperoned from an inactive to an active kinase using a five-protein system. Notably, however, the proteins required to chaperone Chk1 differ from those required to chaperone steroid receptors. Collectively, our results identify the minimal complement of chaperone and co-chaperone proteins essential to chaperone a protein kinase, demonstrate a novel model system to dissect the chaperoning reaction for this import class of Hsp90 clients, and suggest a model for the process of chaperoning Hsp90-dependent kinases.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Casein kinase-2 (CK2), cycloheximide, phosphochreatine, and creatine phosphokinase were purchased from Sigma. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was generously provided by Kossan Biosciences. [γ-32P]ATP (4500 Ci/mmoll was purchased from MP Biomedicals (formerly ICN Radiochemicals). Redivue1-[35S]methionine (>1000 Ci/mmoll was from Amersham Biosciences (GE Healthcare). Mouse monoclonal antibodies that recognize various antigens were obtained as follows: anti-Cdc37 (MA3–029) was from Affinity BioReagents and used for immunoblotting according to the supplier’s instructions; anti-HA (MMS-101P) was from BabCO.

The abbreviations used are: Hsp, heat shock protein; CK2, casein kinase-2; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; GST, glutathione S-transferase; FL, full-length; HA, hemagglutinin.

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(Covance Research Products) and used for immunoprecipitations as directed by the supplier; anti-Chk1 (sc-7898), anti-Raf-1 (sc-133), and anti-HA-conjugated agarose (sc-7292AC) were from Santa Cruz Biotechnology and used as directed by the supplier; anti-Hsp90 (H9010) and anti-Hsp70 (B870) have been previously described (15, 16).

Plasmid Construction—HA-tagged full-length human Chk1 was PCR amplified from a previously described plasmid (17, 18), and the resulting PCR fragment was digested with EcoRI and XbaI and subcloned into pcDNA3 (Invitrogen) to produce pcDNA3-hChk1-HA², a plasmid that expresses a C-terminal tagged Chk1 in mammalian cells or in in vitro transcription/translation reactions. HA-tagged human Chk1 (encoding amino acids 1–265 of Chk1) was produced by PCR amplifying amino acids 1–265 of Chk1. The PCR product was then digested with EcoRI and Ascl and ligated into EcoRI- and Ascl-digested pcDNA3-hChk1-HA². These restriction enzymes excised full-length Chk1 from the pcDNA3-HA² backbone, allowing replacement with the Chk1(1–265) fragment. The resulting plasmid (pcDNA3-Chk1(1–265)-HA²) expressed truncated Chk1 that was C-terminal-tagged (identical to the tag in pcDNA3-hChk1-HA²) in mammalian cells or in in vitro transcription/translation reactions. Glutathione S-transferase (GST)-tagged full-length Chk1 (GST-Chk1(FL)) or GST-Chk1(FL)-His₆ expression vectors were generated using a PCR-based strategy to fuse GST to the N terminus of full-length Chk1 (and the His₆ epitope for the dually tagged Chk1). The resulting PCR fragment was then cloned into pET-21(+) (Novagen) to produce pET-21(+)–GST-Chk1(FL). Similarly truncated GST-tagged Chk1 fusions that express the conserved kinase domain (residues 1–265) or the kinase domain plus a 24-residue linker region (residues 1–289) were also generated (GST-Chk1(1–265) and GST-Chk1(1–289), respectively). The Escherichia coli expression vector for the His₆ N-terminal-tagged Cdc37 S13A was constructed using a PCR-based strategy to clone wild-type Cdc37 into pET24(+), followed by incorporation of the S13A mutation using the QuikChange kit (Stratagene). All constructs were sequenced to verify accuracy of PCR amplification. The estrogen receptor expression plasmid was obtained from P. Webb (University of California San Francisco, CA).

Cross-linking Antibodies to Protein A-Sepharose—Antibodies to Hsp90 and Chk1 were covalently cross-linked to protein A-Sepharose beads (Sigma) using 20 mg/ml dimethyl pimelimidate (Sigma) in 0.2 M sodium borate, pH 9.0, for 30 min. The reaction was quenched by washing the beads with 0.2 M ethanolamine (Sigma). Antibody cross-linked beads were stored in 20 mM Tris, 50 mM KCl, and 5 mM MgCl₂ at 4 °C.

Protein Purification—Purified GST-Cdc25C (residues 200–256) was prepared as described (18). Hsp90 and Hsp70 were overexpressed in SF9 cells infected with baculovirus as described by Alnemri and Litwack (19), and purified as previously described (20, 21). Bacterially expressed Chk1 and the His₆ tag were expressed in Escherichia coli strain M15[pRep4] using a plasmid obtained from R. Matts (Oklahoma State University, Stillwater, OK). The fusion protein was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (1 mM IPTG) at 30 °C. Hsp90-Chk1 was expressed in BL21(DE3) by induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h at 17 °C. Following induction, cells were lysed by sonication in 20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.5 mg/ml lysozyme, and a protease inhibitor mixture (Complete, EDTA-free, Roche). The lysate was cleared by centrifugation and the supernatant was applied to a TALON metal affinity resin (Clontech). Following extensive washing with 20 mM Tris, pH 8.0, 500 mM NaCl, and 5 mM imidazole, the bound Chk1 was eluted from the column with an imidazole gradient. Fractions containing Cdc37 were pooled, dialyzed into 20 mM Tris, pH 7.5, 50 mM KCl, and 2 mM dithiothreitol, and stored at −80 °C.

The expression of GST-Chk1(FL)-His₆ in Rosetta(DE3) pLysS (Novagen) was induced in 4 liters with 1.0 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C. Cells were lysed in 20 mM Tris, pH 8.0, 500 mM NaCl, 15 mM imidazole, and 1 mM 2-mercaptoethanol containing 40 µg/ml aprotinin, 20 µg/ml pepstatin, 20 µg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Cleared cell lysate was adjusted to 1% Triton X-100 and incubated with 0.5 ml of Ni²⁺-NTA-agarose (Qiagen) at 4 °C for 2 h, washed extensively in lysis buffer containing 20 mM imidazole and 1% Triton X-100 (but no protease inhibitors), and bound protein was eluted with 20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM Triton X-100, and 1 mM imidazole. Eluted proteins were then incubated with 0.5 ml of glutathione-agarose (BD Biosciences) at 4 °C for 2 h. The beads were then washed extensively with 20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol, and 1% Triton X-100, followed by washing with 20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, and 10 mM dithiothreitol. The beads (with bound Chk1) were then used for chaperoning assays.

Chk1 Chaperoning Assays—Inactive ³⁵S-labeled, HA-tagged Chk1 was expressed in vitro in the presence of 10 µM 17-AAG in a 25-µl reaction using the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the supplier’s instructions for 1 h at 30 °C. Chk1 was immunoprecipitated from the reaction using anti-HA-conjugated agarose in 1 ml of wash buffer (20 mM Tris-HCl, pH 7.4, 0.27 M sucrose, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 5 mM NaF, 0.1% 2-mercaptoethanol, 20 mM β-glycerophosphate, and 1 mM sodium orthovanadate) containing 0.5 mM NaCl, and freshly added 20 µg/ml aprotinin, 10 µg/ml pepstatin, 20 µg/ml leupeptin. Alternatively, purified GST-Chk1(1–265) or soluble bacterial lysates were bound to GSH-agarose in the above buffer. Immobilized Chk1 was washed 3 times with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 1 mM dithiothreitol). Pellets of Chk1-bound resin (15 µl) were incubated in a 100-µl reaction containing rabbit reticulocyte lysate (Green Hectares) with an energy regeneration system consisting of 10 mM phosphocreatine and 50 µM creatine phosphokinase. When activated with purified chaperone proteins, 0.25 µg of resin-bound GST-Chk1 protein was incubated in a 100-µl reaction with various amounts of Hsp90, Hsp70, Ydj1, Hop, p23, Cdc37, and CK2 in 20 mM Tris, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 5 mM ATP, 10 mM dithiothreitol (chaperoning buffer). Reactions were gently mixed every 5 min and incubated at 30 °C for 1 h or as otherwise indicated. At the end of the chaperoning incubation, bead pellets were washed 4 times with wash buffer supplemented with 1.0 M
NaCl, and 0.025% SDS and 4 times with kinase buffer in preparation for subsequent kinase assays.

**Immunodepletion Assays**—GST-Chk-(1–265) (1 μg) was incubated at 30 °C for 90 min with 2 μg of Hsp90, 20 μg of Hsp70, 4 μg of Ydj1, 4 μg of Cdc37, 5 μg of Hsp, 5 μg of p23, and 0.14 μg of CK2 in a 200-μl reaction in chaperoning buffer. The reaction mixture was split into two equal parts, and each portion was incubated with 20-μl pellets of Hsp90 or Chk1 antibody cross-linked beads for 1 h at 4 °C. Supernatants were then subjected to a second identical round of immunodepletion. Finally, all supernatants, whether previously Hsp90 or Chk1 depleted, were incubated with anti-Chk1 cross-linked beads for 1 h at 4 °C. All pellets were washed once with wash buffer supplemented with 0.5 mM NaCl and twice with kinase buffer in preparation for subsequent kinase assays. Additionally, a fraction of each of the final supernatants was saved for SDS-PAGE and immunoblotting.

**Chk1 Kinase Assays**—Chk1 activity was determined similarly to a previously described procedure (25) by incubating bead-bound Chk1 with [γ-32P]ATP and the Chk1 substrate GST-Cdc25C (residues 200–256) at 30 °C for 10 min. Kinase reactions were terminated with the addition of 4× SDS-PAGE sample buffer and heated at 100 °C for 10 min. Reactions were then separated by SDS-PAGE (12.5% SDS gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore). Membrane-bound radiolabeled proteins were detected and quantitated using a Storm 840 PhosphorImager (Amersham Biosciences).

**Immunoprecipitations**—HeLa cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 2 mM l-glutamine. Cells were plated in 100-mm dishes, grown to 90% confluence, and lysed on ice for 10 min in 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.27 M sucrose, 1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 mM microcystin-LR, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 20 μg/ml leupeptin. Lysates were clarified by centrifugation for 5 min at 15,000 × g of cell lysate protein. For comparison, immunoprecipitations were run alongside serial dilutions of whole cell lysate. Samples were separated by SDS-PAGE (10% gel) and immunoblotted for Chk1, Raf-1, and Hsp90. Mouse IgG TrueBlot was used as a secondary antibody for the Chk1 immunoblot. An asterisk denotes a nonspecific band that is detected in the Chk1 blot of Hsp90 immunoprecipitates. The dashed line indicates the juxtaposition of non-adjacent lanes from the same immunoblot (to remove an intervening protein standard lane). IP, immunoprecipitation. The experiment was repeated 3 times with similar results.

Hsp90 immunoprecipitates in control cells or in cells treated with hydroxyurea, a potent inhibitor of replication and activator of Chk1. (Note that the band marked with an asterisk did not co-migrate with Chk1.) This discrepancy with our previous finding, that Chk1 immunoprecipitates contained Hsp90, is likely because of the fact that immunoblotting with the anti-Hsp90 antibody is more sensitive than immunoblotting with the anti-Chk1 antibody (34). Nonetheless, given that 1000 μg of cell lysate protein were immunoprecipitated and that we could readily detect Chk1 in as little as 10 μg of cell lysate, the simplest explanation of these results is that only a small percentage of Chk1 is stably associated with Hsp90 in exponentially growing cells, a result that is similar to what has been reported for other Hsp90 client kinases (40, 41).

**RESULTS**

Some Hsp90 clients, such as the steroid receptors, are constitutively associated with and maintained in a functional conformation by Hsp90 (26–29). In contrast, other Hsp90 clients only transiently associate with the chaperoning machinery and are released from Hsp90 in a functional form (30–33). Our previous results showed that Chk1 immunoprecipitates contained Hsp90 and that short-term inhibition of Hsp90 did not affect Chk1 activity (34). Together, these results suggested that Chk1 interacts transiently with Hsp90 during the chaperoning reaction and is then released as a functional kinase. To test this prediction and gain further insight into the interplay between Chk1 and Hsp90, we examined what fraction of Chk1 is associated with Hsp90 by immunoprecipitating Hsp90 and blotting for Chk1 (Fig. 1). Because Chk1 migrates very near immunoglobulin G heavy chain, we covalently cross-linked the anti-Hsp90 monoclonal antibody to protein A-Sepharose for this experiment. We first demonstrated that covalent coupling did not disrupt the ability of Hsp90 to co-precipitate Raf-1, a bona fide Hsp90 client (35–39). However, despite repeated attempts we did not observe Chk1 in the

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![Figure 1. Endogenous Chk1 is undetectable in Hsp90 immunoprecipitates.](image)

Hela cells were grown to 90% confluence in 100-mm dishes and treated with 10 μm hydroxyurea (HU) 1 h before lysis. Hsp90 or HA were immunoprecipitated from lysates (1 mg of protein). For comparison, immunoprecipitations were run alongside serial dilutions of whole cell lysate. Samples were separated by SDS-PAGE (10% gel) and immunoblotted for Chk1, Raf-1, and Hsp90. Mouse IgG TrueBlot was used as a secondary antibody for the Chk1 immunoblot. An asterisk denotes a nonspecific band that is detected in the Chk1 blot of Hsp90 immunoprecipitates. The dashed line indicates the juxtaposition of non-adjacent lanes from the same immunoblot (to remove an intervening protein standard lane). IP, immunoprecipitation. The experiment was repeated 3 times with similar results.

To assess whether Hsp90 played a role in producing catalytically active Chk1, we developed cell-free systems that generated catalytically active Chk1.

For our first cell-free system, we took advantage of the fact that rabbit reticulocyte lysate, a rich source of chaperoning proteins, has been used previously to chaperone Hsp90 client kinases (1). In vitro transcription/translation reactions were programmed with empty vector or a vector that expresses HA-tagged full-length Chk1 and immunoprecipitated with an anti-HA antibody conjugated to agarose beads. Following washing, the precipitates were subjected to kinase assays by incubating with [γ-32P]ATP and the GST-Cdc25C substrate. As shown in Fig. 2A, in the reactions programmed with empty vector, no [35S]-labeled Chk1 was visible (upper panel), and only background kinase activity was detected (lower panel and graph, lanes 1–4). In the reactions programmed with the Chk1 expression vector, [35S]-labeled Chk1 was clearly present and the translated kinase exhibited robust kinase activity (lane 5).

To assess whether Hsp90 played a role in producing catalytically active Chk1 in this system, we added the Hsp90 inhibitor 17-AAG (42). When Chk1 was translated in the presence of 17-AAG, Chk1 was produced; however, only background kinase activity was present (lane 6). We consistently noticed that 17-AAG reduced the amount of [35S]-labeled Chk1, raising the possibility that the reduced kinase activity was the result of diminished amounts of Chk1 (cf. lanes 5 to 6–8). To adjust for differing amounts of Chk1, we calculated the specific activity of Chk1 by dividing the counts of [32P] substrate by the counts of [35S] Chk1. When translated in the absence of 17-AAG, Chk1 had a specific activity of 2.0. In contrast, when translated in the presence of 17-AAG, Chk1 specific activity was 0.4, a 5-fold reduction. Collectively, these results demonstrated that Hsp90 is required for Chk1 to acquire
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kinase activity in an in vitro translation reaction and showed that Chk1 could be chaperoned in an in vitro system.

Given that we could produce unchaperoned Chk1 by translating in the presence of 17-AAG, we then asked whether Hsp90 was required co-translationally or whether Hsp90 could chaperone the kinase after it had been produced in an inactive form. Following translation in the presence of 17-AAG, the inactive Chk1 was immunopurified and incubated with fresh reticulocyte lysate either in the presence or absence of 17-AAG (Fig. 2A). The inactive Chk1 acquired catalytic activity (specific activity = 3.7) when incubated with fresh reticulocyte lysate (lane 7), whereas in the presence of 17-AAG (lane 8) the acquisition of Chk1 activity (specific activity = 1.0) was attenuated 3.7-fold. These results indicate that Hsp90 plays a major role in chaperoning Chk1; however, they also suggest that Hsp90-independent chaperoning of Chk1 may occur in this system.

Although the in vitro transcription/translation system was capable of generating unchaperoned Chk1, we sought a more tractable method to produce larger quantities of purified, unchaperoned Chk1. Because eukaryotic and bacterial chaperone systems differ, we reasoned that Chk1 expressed in E. coli would be poorly chaperoned. Indeed, the majority of bacterially expressed GST-Chk1 (full-length, FL) was found in inclusion bodies (data not shown), suggesting that the kinase was not correctly chaperoned in the absence of eukaryotic chaperoning machinery. Correspondingly, the small amount of soluble GST-Chk1 (FL) had a very low level of catalytic activity (Fig. 2B). Incubation of this bacterially expressed GST-Chk1(FL) with rabbit reticulocyte lysate resulted in a large increase in kinase activity. Notably, the activation was also inhibited by 17-AAG, demonstrating that the bacterially expressed GST-Chk1 is chaperoned in an Hsp90-dependent manner.

SDS-PAGE analysis of the GST-Chk1(FL) used in the above experiment demonstrated that very little full-length fusion protein was present in this preparation (Fig. 2C). This raised two concerns. First, because the protein was not homogeneous, it was unclear whether full-length or a fragment of Chk1 was being chaperoned. Second, the yield of fusion protein was very low, which would preclude a detailed biochemical analysis of the chaperoning. To circumvent these problems, we created two truncated GST-Chk1 fusion proteins that fused the conserved kinase domain (residues 1–265) or the kinase domain plus an additional 24-amino acid linker (residues 1–289) of Chk1 to GST. These fragments, when expressed in insect cells, are catalytically active (43). Again, the majority of GST-Chk1(1–265) and GST-Chk1(1–289) was found in inclusion bodies when expressed in E. coli (data not shown). Notably, however, the soluble fusion proteins were more homogeneous than the full-length preparation (Fig. 2C) and were produced with better yield (data not shown). When assayed for kinase activity, all three fusion proteins exhibited low levels of catalytic activity as isolated from the bacteria, perhaps as a consequence of weak or incomplete chaperoning in E. coli (Fig. 2D). Incubation of the fusion proteins with reticulocyte lysate dramatically increased kinase activity of all three proteins. Because it is unclear whether a fragment or the full-length GST-Chk1 gave rise to active Chk1 kinase, we did not attempt to quantitate the fusion proteins for this analysis. Instead, to determine which GST-Chk1 would be the best substrate for further analysis of Chk1 chaperoning, we calculated the fold increase in activity by dividing the 32P incorporated into GST-Cdc25C by each GST-Chk1 after incubation with reticulocyte lysate by the activity of GST-Chk1 activity before incubation with reticulocyte lysate. This analysis revealed that GST-Chk1(FL), GST-Chk1(1–265), and GST-Chk1(1–289) were increased by 50-, 50-, and

![Figure 2](image.png)

Chk1 chaperoning requires Hsp90. A, in vitro transcription/translation reactions containing [35S]methionine were programmed with empty vector (EV) or pcDNA3-Chk1-HA2 (full-length Chk1) and performed in the presence or absence of 10 μM 17-AAG. The reaction products were then immunoprecipitated with anti-HA antibodies, and the precipitates were used directly for kinase reactions (lanes 1, 2, 5, and 6) or were further incubated with 100 μl of rabbit reticulocyte lysate (RRL) (lanes 2 and 7) or with reticulocyte lysate containing 10 μM 17-AAG (lanes 4 and 8) for 30 min at 30 °C. Following incubation in RRL, the precipitates were washed and subjected to kinase assays in parallel with the samples in lanes 1, 2, 5, and 6. Washed immunoprecipitates were incubated with GST-Cdc25C and [γ-32P]ATP in kinase buffer, separated by SDS-PAGE (12.5% gel), and transferred to Immobilon-P. Radiolabeled Chk1 (upper panel) and GST-Cdc25C were detected (middle panel) and quantitated (graph) using a PhosphorImager. The experiment was repeated 4 times with similar results. B and D, lysates from E. coli transformed with empty vector, GST-Chk1(FL) (B), GST-Chk1(1–265) (D), or GST-Chk1(1–289) (D) expression vectors were incubated with GSH-agarose to recover GST-Chk1 proteins. The GSH-agarose beads were then washed and incubated with GST-Cdc25C and [γ-32P]ATP in kinase buffer. Reaction products were separated by SDS-PAGE, transferred to Immobilon-P, and imaged by PhosphorImager analysis. C, fold activation for GST-Chk1(FL), GST-Chk1-(1–265), and GST-Chk1-(1–289) were separated by SDS-PAGE and the gel was stained with Coomassie Blue. Masses (kDa) of standards are indicated on the left-hand side of the figure.
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6-fold, respectively. Because the GST-Chk1-(1–265) fusion exhibited the greatest increase in activity, was the most homogeneous, and was produced with the greatest yield, this protein was used to further characterize the Chk1 chaperoning reaction using purified chaperones.

Chk1 Can Be Chaperoned with Purified Chaperone Proteins—The experiments described above demonstrated that we had produced a plentiful source of unchaperoned Chk1 that was robustly chaperoned to an active kinase in an Hsp90-dependent reaction in reticulocyte lysates. With this system in hand, we then asked whether GST-Chk1-(1–265) could be chaperoned using purified proteins. The three core chaperones, Hsp90, Hsp70, and Ydj1 (yeast Hsp40), alone could not chaperone Chk1 into an active kinase (Fig. 3A). The addition of Hop and p23 to the three-protein mixture also did not result in active Chk1, demonstrating that Chk1 chaperoning differs substantially from chaperoning of the steroid receptors. Because Cdc37 has been implicated as an essential component in the chaperoning of many kinases, we added Cdc37 to the mixture; however, no active Chk1 was recovered. Recent reports have shown that Cdc37 is phosphorylated by CK2 on Ser13, a phosphorylation required for Cdc37 to interact with kinase clients (44, 45). Because the Cdc37 used in these assays was isolated from bacteria, it likely would not be phosphorylated on Ser13. Therefore, we added CK2 to the chaperoning reaction and asked whether this kinase affected Chk1 chaperoning. As shown in Fig. 3A the addition of CK2 to a chaperoning reaction containing Hsp90, Hsp70, Ydj1, and Cdc37 increased Chk1 activity by 600-fold. Taken together, these results demonstrate that these five proteins constitute the minimal system required to chaperone Chk1. A slight further stimulation (0.25-fold) occurred when both Hop and p23 were included in the reaction. Importantly, phosphorylation of the GST-Cdc25C substrate only occurred when recombinant Chk1-(1–265) was included in the chaperoning reaction, thus demonstrating that substrate phosphorylation was not because of CK2 or other contaminating kinases nonspecifically binding the glutathione-agarose.

Chk1 Chaperoning Requires CK2 Phosphorylation of Cdc37—To further explore the role of Cdc37 phosphorylation by CK2 in the chaperoning reaction, we asked whether mutation of Ser13, the reported CK2 phosphorylation site on Cdc37, affected Chk1 chaperoning in this purified system. Consistent with previous reports (44, 45), mutation of Cdc37 Ser13 to Ala prevented CK2-mediated phosphorylation of Cdc37 (Fig. 3B). Moreover, unlike wild-type Cdc37, the Cdc37 S13A mutant did not support Chk1 chaperoning in this system (Fig. 3C). These results demonstrate that Cdc37 phosphorylation on Ser13 is essential for Cdc37 function in this in vitro system, and they further indicate that the phosphorylation of the GST-Cdc25C substrate is mediated by chaperoned Chk1 and not CK2.

Hop Stimulates Hsp90-dependent Chk1 Chaperoning—Hop and p23 are co-chaperones that stimulate chaperoning of the steroid receptors.
The results in Fig. 3A showed that Chk1 chaperoning was slightly stimulated when Hop and p23 were included in the reactions, suggesting that these co-chaperones might also enhance Chk1 chaperoning. We first tested whether p23 affected Chk1 chaperoning using a variety of conditions; however, p23 did not stimulate the chaperoning under any conditions tested (data not shown and see Fig. 5A). In contrast, Hop modestly (2-fold) stimulated Chk1 chaperoning when 10 \( \mu \text{g} \) of Hsp90 was used in the reaction (Fig. 4). Because Hop has been implicated in recruiting Hsp90 to Hsp70-associated clients, we reasoned that Hop might have a greater stimulatory effect at low concentrations of Hsp90. Consistent with this prediction, we found that Hop dramatically stimulated Chk1 chaperoning when Hsp90 was limiting.

Additional titration experiments demonstrated that the amount of Cdc37 included in the chaperoning reaction could be reduced from the amount used in the previous experiments without adversely affecting Chk1 activity (data not shown). Based on these results, subsequent experiments were performed using a 100-\( \mu \text{l} \) chaperone protein mixture containing 1 \( \mu \text{g} \) of Hsp90, 2 \( \mu \text{g} \) of Cdc37, 10 \( \mu \text{g} \) of Hsp70, 2 \( \mu \text{g} \) of Ydj1, 2.5 \( \mu \text{g} \) of Hop, 2.5 \( \mu \text{g} \) of p23, and 0.07 unit of CK2.

**Role of Individual Chaperones and Co-chaperones**—We sought to further understand the process of Chk1 chaperoning by removing individual chaperone and co-chaperone proteins from the incubation and examining the effects on Chk1 activity (Fig. 5A) under conditions (1 \( \mu \text{g} \) of Hsp90) where Hop maximally stimulated the chaperoning reaction (see Fig. 4). When all proteins were included in the reaction, robust Chk1 activity was observed (lane 2). As previously mentioned, leaving p23 out of the reaction had only a very modest effect on Chk1 kinase activity (lane 9). In sharp contrast, however, when any other protein was absent from the chaperoning reaction, background levels of Chk1 activity were observed, indicating that all these proteins are essential for Chk1 chaperoning in vitro.

It is possible that chaperoning requirements for Chk1-(1–265) and full-length Chk1 differ. To address this possibility, we devised a method to produce a small amount of un-chaperoned full-length Chk1 in *E. coli* by fusing a His\(_6\) tag onto the C terminus of GST-Chk1(FL). Following sequential purification using the N-terminal GST and C-terminal His\(_6\) tags, an intact GST-Chk1(FL)–His\(_6\) protein was produced (Fig. 5C). As isolated from *E. coli*, this protein exhibited very low kinase activity, similar to what was observed with GST-Chk1(1–265) (Fig. 5B). Incubation of GST-Chk1(FL) with the purified chaperones also increased activity. Although the -fold activation was lower using the full-length protein, in vitro chaperoning of full-length Chk1 required the identical cohort of purified proteins as did Chk1-(1–265).

**Time Course of Chk1 Chaperoning and Association of Chaperone with Chk1**—To identify the optimal time point for Chk1 chaperoning reactions, we performed a time course experiment in which Chk1 activity was assayed after various incubation times (Fig. 6). Because Cdc37 must be phosphorylated by CK2 for chaperoning, we preincubated the chaperone protein mixture with CK2 for 1 h to pre-phosphorylate Cdc37 prior to adding the chaperone mixture to Chk1 (1–265). Under these conditions, Chk1 chaperoning occurred gradually, with kinase activity reaching a plateau at 60 min. A similar time course of chaperoning was seen when Chk1 was chaperoned with rabbit reticulocyte lysate (data not shown).

To gain additional insight into the chaperoning reaction, we examined the association of chaperones during this time course by immunoblotting the membrane to identify Chk1-associated proteins. Despite repeated efforts, and perhaps as a result of the stringent washes, we did not observe stable binding of Hop, Ydj1, or p23 to Chk1 (Fig. 6). In contrast, Hsp70, Hsp90, and Cdc37 associated stably with Chk1. Hsp70 associated with Chk1 very rapidly, with the amount of associated Hsp70 decreasing over time, suggesting that Hsp70 dissociated as the complex matured. Hsp70 binding was quickly followed by Cdc37 association; however, unlike Hsp70, the amount of bound Cdc37 increased throughout the time course. Of these three chaperone proteins, Hsp90 was the last to bind Chk1, and its binding also increased throughout the time course.
The C-terminal Regulatory Domain of Chk1 Is Required for Release from Hsp90—Given that we were unable to co-precipitate Chk1 with Hsp90 in cell lysates, the robust binding of Hsp90 to Chk1 in the purified chaperoning system was somewhat surprising. Therefore, we then asked whether active Chk1 remains bound to Hsp90 or if it is released from the chaperone complex using the experimental approach outlined in Fig. 7A. To address this question, GST-Chk1-(1–265) was activated in a 200-μl reaction. The reaction was then split into two equal parts, which were analyzed for total Chk1 kinase activity (Sample 2) and Hsp90-associated kinase activity (Sample 1). To assess total Chk1 kinase activity, GST-Chk1-(1–265) was immunoprecipitated from Sample 2 (three rounds of immunodepletion; Samples A–C). Fig. 7B shows that the immunoprecipitations removed nearly all the GST-Chk1-(1–265) from the reaction (cf. Sample 2A and 2C) and that Hsp90 was associated with this pool of Chk1 (Sample 2A). In parallel, the other half of the chaperoning reaction (Sample 1) was immunoprecipitated with anti-Hsp90 antibodies (2 rounds of immunoprecipitation; Samples 1A–C), which removed all the Hsp90 (cf. Samples 1A–1C). Following the second Hsp90 immunodepletion of Sample 1, the GST-Chk1-(1–265) remaining in the supernatant was recovered by immunoprecipitating with anti-Chk1 antibodies (Sample 1C). No Hsp90 was observed in the Chk1 recovered after Hsp90 immunodepletion (Sample 1C), demonstrating that this pool of Chk1 is not bound to Hsp90. All the immunoprecipitates were then subjected to kinase assays. When Chk1 was directly recovered from the soluble chaperoning reaction, abundant catalytic activity was observed (Sample 2A). Likewise, the Chk1 that co-precipitated with Hsp90 (Sample 1A) contained a similar level of kinase activity. Surprisingly, however, the Chk1 that was not associated with Hsp90 (Sample 1C) had only background levels of catalytic activity, suggesting that the system either: 1) lacks additional factors that are required to release chaperoned Chk1 or 2) the C-terminal regulatory domain, which has been deleted in Chk1-(1–265), plays a role in Chk1 chaperoning or stability.

To distinguish between these possibilities, we compared the interactions of full-length Chk1 and Chk1-(1–265) with Hsp90 following translation in reticulocyte lysates. Full-length HA-tagged Chk1, HA-tagged Chk1-(1–265), and, as a control, estrogen receptor, were expressed in coupled transcription/translation reactions for 30 min (Fig. 7, C and D). Translation was then blocked by the addition of cycloheximide so that chaperoning reactions could be followed over time without the translation of additional proteins. For analysis, samples were removed at 0, 30, and 60 min after the inhibition of translation, and each sample was divided; one portion was analyzed directly by separation on SDS-PAGE and the other portion was assayed for interaction of the client with Hsp90 by immunoprecipitating with anti-Hsp90 monoclonal antibody. Like the estrogen receptor, which constitutively associates with Hsp90 in intact cells, Chk1-(1–265) also continued to interact with Hsp90 during the 60-min time course. In contrast, full-length Chk1 was released from Hsp90, a result similar to what is observed with endogenous Chk1 in intact cells. Taken together, these results suggest that the C-terminal regulatory domain (that has been removed in Chk1-(1–265)) plays a role in Chk1 chaperoning.

**DISCUSSION**

Much of our understanding of kinase client chaperoning has been gleaned from genetic studies in yeast and biochemical analyses in complex systems. However, no Hsp90 protein kinase clients have been successfully chaperoned using purified systems, thus hampering our efforts to understand, at the molecular level, the chaperoning requirements for protein kinases. As a first step toward filling this gap we report the identification of a reproducible source of unchaperoned Chk1 and show that this Chk1 can be chaperoned in a purified system consisting minimally of Hsp70, Hsp40, Hsp90, Cdc37, and CK2. Collectively, these studies yield novel insights into the proteins required to chaperone bacterially expressed Chk1 in vitro and provide a powerful system to further probe the biochemical events required to chaperone this important class of Hsp90 clients.

Although only a few clients have been chaperoned in vitro with purified proteins (6–9, 13, 46, 47), comparisons of the chaperone requirements for these clients provide additional insight into this Hsp90-dependent process. The steroid receptors, which are the most thoroughly studied clients, are chaperoned through a multistep process that requires 5 purified proteins (reviewed in Ref. 1). Receptor chaperoning begins with the binding of Hsp70 and Hsp40 and stimulation of the ATPase activity of Hsp70 by Hsp40. This complex then binds Hop, which in turn facilitates the recruitment of Hsp90. ATP binding to Hsp90 then induces a conformational shift in Hsp90, triggering the recruitment of p23 and the release of Hsp40, Hsp70, and Hop. The receptor, bound to Hsp90 and p23, is then competent to bind hormone. Likewise, duck hepatitis virus B reverse transcriptase and human telomerase (12, 46) are chaperoned by the same 5-protein system.

Our analysis of Chk1 chaperoning revealed significant differences in the chaperoning requirements for this kinase. As has been observed with the steroid receptors, reverse transcriptase, and telomerase, Chk1 required the core chaperones Hsp70, Hsp40, and Hsp90. Differences arose, however, when we analyzed the role of p23, a small highly charged protein that stabilizes ATP-bound Hsp90 (48). Whereas steroid receptors, reverse transcriptase, and telomerase chaperoning is stimulated, in some cases dramatically, by p23, chaperoning of Chk1-(1–265) was not enhanced in our system under any conditions tested. A similar conclusion for the role of p23 in v-Src chaperoning has been reached using

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2 S. J. H. Arlander, unpublished data.
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Figure 7: Active Chk1 is associated with Hsp90. A, schematic of experimental design. B, 1 μg of GST-Chk1-(1–265) was incubated with 2 μg of Hsp90, 20 μg of Hsp70, 4 μg of Ydj1, 4 μg of Cdc37, 5 μg of Hsp, 5 μg of p23, and 0.14 units of CK2 at 30 °C for 90 min. At the completion of the chaperoning reaction, the reaction was divided into two 100-μl samples. For Sample 1, two rounds of Hsp90 immunodepletion were performed with antibody cross-linked protein A-Sepharose. The GST-Chk1-(1–265) remaining in the supernatant was then recovered with anti-Chk1 antibody cross-linked to protein A-Sepharose. For Sample 2, Chk1 was immunodepleted by three rounds of incubation with anti-Chk1 antibody cross-linked to protein A-Sepharose. The GS-agarose beads were then incubated with [γ-32P]ATP and GST-Cdc25C substrate in kinase buffer. Kinase reaction products were separated by SDS-PAGE, transferred to Immobilon-P, and imaged by PhosphorImager analysis (bottom panel). Hsp90 (upper panel) and Chk1 (middle panel) were detected by immunoblotting. The supernatants that remained after the 3 rounds of immunoprecipitation were immunoblotted for Hsp90 to show that Hsp90 was removed from Sample 1 but not Sample 2 (right panel). C, in vitro transcription/translation reactions containing [35S]methionine were programmed with vector encoding the estrogen receptor (ER), pcDNA3-hChk1-HA2 (Chk1 FL), or pcDNA3-Chk1–265-HA2 (Chk1–265). After 30 min, translation was terminated by the addition of 100 μg/ml cycloheximide. Samples were then removed at 0, 30, and 60 min after cycloheximide addition and divided. One portion of the divided sample was prepared for SDS-PAGE (labeled Translation), and the other portion was immunoprecipitated with anti-Hsp90 monoclonal antibody H9010 (Hsp90 IP), washed, and boiled in SDS-PAGE sample buffer. Samples were then separated by SDS-PAGE (10% gel) and transferred to Immobilon P. Radiolabeled proteins were imaged by PhosphorImager analysis, and Hsp90 was detected by immunoblotting. D, the experiment shown in C was repeated (n = 3 ± S.D.) and percent initial bound was calculated by dividing the amount of Hsp90-bound 35S in each sample by the amount of Hsp90-bound 35S in the 0-min time point for that protein.

Yeast as a model system. Deletion of the Saccharomyces cerevisiae homolog of the p23 gene (Sba1) has only a minimal effect on cell growth and a very modest effect on c-Src activity (49, 50). In contrast, p23 plays an important role in chaperoning the Hsp90 client Gcn2 in yeast (51). Thus, even though p23 plays an important stimulatory role in chaperoning some clients, studies in yeast, and the present study, suggest that not all clients require this protein.

The chaperone Hop is also important for chaperoning other clients in
purified systems. A large body of data supports a model in which Hop recruits Hsp90 to Hsp70-bound clients, suggesting that Hop plays a critical role in chaperoning (reviewed in Ref. 52). On the other hand, studies in S. cerevisiae have shown that the yeast Hop1 gene (Sti1) is not essential for (but does play a stimulatory role in) the chaperoning of the Ste11 kinase, an Hsp90 client (53). We found that Hop was not required to chaperone Chk1 when Hsp90 levels were high. In contrast, when Hsp90 was limiting, Hop dramatically stimulated the reaction. This observation raises the intriguing possibility, which will be tested in future studies, that the requirement for Hop might vary with the level of Hsp90 expression in the cell.

We also examined the role of Cdc37 in Chk1 chaperoning. Previous analyses of the role of Cdc37 in Hsp90 chaperoning have been limited to reticulocyte lysates, yeast genetic systems, and studies in intact mammalian cells. The present studies demonstrate the absolute requirement for Cdc37 to chaperone Chk1 in a purified chaperone system. One proposed role for Cdc37 is to recruit Hsp90 to the client by simultaneously binding the client and Hsp90 (54). Published studies have shown that the interaction between Cdc37 and client depends on CK2-mediated phosphorylation of Cdc37 (44, 45). Consistent with these findings, the present studies show that Chk1 chaperoning requires CK2 and the phosphorylation of Cdc37 on Ser13; however, it is possible that the activities of other chaperones, co-chaperones, or even Chk1 itself might also be affected by CK2 phosphorylation.

Some Hsp90 clients, such as the progesterone receptor, require continuous association with Hsp90 to maintain a functional form, whereas other clients are chaperoned during a transient interaction with Hsp90 and are then released as functional and stable proteins. Hsp90 kinase clients fall primarily in the latter category, although there are clear differences in the degree of association with Hsp90. For example, a significant portion of Rous sarcoma virus v-Src is complexed with Hsp90 and Cdc37, and the interaction is readily detected by co-immunoprecipitation (30, 55–57). Similarly, about one-third of the Akt pool is associated with Cdc37 and this associated pool is catalytically active (58). On the other hand, like the interaction of Chk1 with Hsp90, the association of wild-type c-Src or Lck with Hsp90 is very difficult, if not impossible, to detect in cell lysates (40, 59). One possible explanation for the limited level of binding is that the interactions are labile and do not withstand the lysis and/or washing conditions required for immunoprecipitation. Alternatively, as has been demonstrated for the Src family kinase Lck (60), the binding of the kinase to Hsp90 might only occur transiently, and once chaperoned, the kinase is released in a stable form that does not require Hsp90 to maintain stability. In the case of Chk1, we favor the latter idea, because the interaction between Hsp90 and full-length Chk1 is readily detected in reticulocyte lysates immediately following translation (Fig. 7, C and D). Then, as the kinase matures in the chaperoning reaction, Chk1 is no longer associated with Hsp90.

Our studies also provide additional insight into the factors that affect the interaction between Hsp90 and Chk1. We were initially surprised to find that the Chk1 fragment (amino acids 1–265) we used for in vitro chaperoning with reticulocyte lysate or purified proteins interacted stably with Hsp90 and other co-chaperones. Notably, the Hsp90-bound Chk1 fragment was catalytically active when chaperoned with purified proteins, and the interaction of Chk1-(1–265) with Hsp90 did not decrease over time in either the purified protein system or the reticulocyte lysate system (Fig. 7C). This was in stark contrast to what was observed with full-length Chk1 in intact cells, where there was no detectable Chk1 in Hsp90 immunoprecipitates (Fig. 1). This discrepancy suggested two possible explanations. On the one hand, the in vitro systems might lack a "release" factor that facilitates the release of Chk1 from the Hsp90 chaperoning machinery. On the other hand, the C-terminal regulatory domain, which is missing in Chk1-(1–265), might play a critical role in the interaction between Chk1 and Hsp90. To determine whether the C terminus was important, we in vitro translated full-length Chk1 and Chk1-(1–265), stopped the translation with cycloheximide, and followed the interaction of each protein with Hsp90 over time during the ensuing chaperone reaction. Full-length Chk1 was rapidly released from Hsp90, consistent with the observation that very little Chk1 is associated with Hsp90 in intact cells. In contrast, in vitro translated Chk1-(1–265) maintained a constant level of association with Hsp90, indicating that the C terminus affects the interaction of Chk1 with Hsp90.

We envision two possible functions for the C terminus during chaperoning. In one, the C terminus might interact with potential release factors that promote the dissociation of Chk1 from Hsp90. Thus, in the absence of the C terminus, Chk1-(1–265) is locked into the Hsp90 machinery, preventing completion of the chaperoning process. In the second possibility, the C terminus might stabilize the catalytic domain following completion of the chaperone cycle. Consistent with this idea, several studies have shown that the C-terminal domain binds and inhibits the Chk1 catalytic domain (43, 61, 62). Thus, in the absence of the C-terminal stabilizing influence, the chaperoned kinase would rapidly unfold, leading to reiterative cycles of chaperoning. Similar findings have been made for Lck and Hck. Phosphorylation-dependent binding of the C terminus of wild-type Lck or Hck to the N-terminal SH2 domain apparently stabilizes the mature kinase so that it no longer requires Hsp90 (59, 63). Correspondingly, if the C-terminal phosphorylation site is mutated, these kinases are less stable and undergo reiterative cycles of chaperoning (59, 63). In a similar manner, Hsp90 maintains steroid receptors in a conformation poised to bind hormone. Following hormone binding the receptor is stabilized and Hsp90 dissociates (reviewed in Ref. 1). Likewise, Hsp90 maintains heme-regulated eukaryotic initiation factor 2 kinase in a conformation that can be phosphorylated and activated by upstream stimuli. Once activated, the kinase is released and no longer requires Hsp90 for stability or catalytic activity (64).

These and other studies support a model for how Hsp90 participates in Chk1 signaling. Following translation, Chk1 enters the Hsp90 chaperoning machinery. Once the kinase domain has been chaperoned, the C-terminal regulatory domain likely interacts with the kinase domain, stabilizing the protein and inhibiting its catalytic activity. The kinase, which then no longer requires Hsp90, is then poised for phosphorylation and activation by ATR in response to genotoxic stress.

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REFERENCES
1. Pratt, W., and Toft, D. (2003) Exp. Biol. Med. 228, 111–133
2. Nollen, E. A., and Morimoto, R. I. (2002) J. Cell Biol. 159, 2899–2916
3. Isaacs, J. S., Xu, W., and Neckers, L. (2003) Cancer Cell 3, 213–217
4. Neckers, L. (2002) Trends Mol. Med. 8, 555–561
5. Goetz, M. P., Toft, D. O., Ames, M. M., and Erlichman, C. (2003) Ann. Oncol. 14, 1169–1176
6. Konno, H., Stenggard, B., Charlesworth, M. C., McMahon, N., and Toft, D. (1998) J. Biol. Chem. 273, 32973–32979
7. Dittmar, K. D., and Pratt, W. B. (1997) J. Biol. Chem. 272, 13047–13054
8. Dittmar, K. D., Demody, D. R., Stancato, L. F., Krishna, P., and Pratt, W. B. (1997) J. Biol. Chem. 272, 21213–21220
9. Dittmar, K. D., Banach, M., Galgani, M. D., and Pratt, W. B. (1998) J. Biol. Chem.
In Vitro Reconstitution of Chk1 Chaperoning

273, 7358–7366
30. Brugge, J., Yonemoto, W., and Darrow, D. (1983)
31. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Toft, D. O. (1996)
32. Yorgin, P. D., Hartson, S. D., Fellah, A. M., Scroggins, B. T., Huang, W., Katsanis, E., Baulieu, E. E. (1984)
33. M., van der Kwast, T. H., Grootegoed, L. J., Berrevoets, C. A., Zegers, N. D., Sanchez, E. R., Toft, D. O., Schlesinger, M. J., and Pratt, W. B. (1985)
34. Johnson, J. L., and Toft, D. O. (1994)
35. Sanchez, E. R., Toft, D. O., Schlesinger, M. J., and Pratt, W. B. (1994)
36. Schulte, T. W., Blagosklonny, M. V., Ingui, C., and Neckers, L. (1995) J. Biol. Chem. 270, 24585–24588
37. Schulte, T. W., Blagosklonny, M. V., Romanova, L., Mushinski, J. F., Monia, B. P., Johnston, J. F., Nguyen, P., Trepel, J., and Neckers, L. M. (1996) Mol. Cell. Biol. 16, 5839–5845
38. Wartmann, M., and Davis, R. J. (1994) J. Biol. Chem. 269, 6695–6701
39. Whitesell, L., Minnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8324–8328
40. Hartson, S. D., and Matts, R. L. (1994) Biochemistry 33, 8912–8920
41. Egorin, M. J., Rosen, D. M., Wolff, J. H., Callery, P. S., Musser, S. M., and Eisenman, J. L. (1998) Cancer Res. 58, 2385–2396
42. Chen, P., Luo, C., Deng, Y., Ryan, K., Register, J., Margosiak, S., Tempczyk-Russell, A., Nguyen, B., Myers, P., Lundgren, K., Kan, C. C., and O'Connor, P. M. (2000) Cell 100, 681–692
43. Hu, J., Toft, D., Darrow, D., and Wang, X. (2002) J. Virol. 76, 269–279
44. Shao, J., Prince, T., Hartson, S. D., and Mats, R. L. (2003) J. Biol. Chem. 278, 38117–38120
45. Miyata, Y., and Nishida, E. (2004) Mol. Cell. Biol. 24, 4065–4074
46. Holt, S. E., Ainsen, D. L., Baur, J., Tesmer, V. M., Dy, M., Ouellette, M., Trager, B. J., Morin, G. B., Toft, D. O., Shuy, J. W., Wright, W. E., and White, M. A. (1999) Genes Dev. 13, 817–826
47. King, F. W., Wawrzynow, A., Hohfeld, J., and Zylucz, M. (2001) EMBO J. 20, 6297–6305
48. Felts, S. J., and Toft, D. O. (2003) Cell Stress Chaperones 8, 108–113
49. Fang, Y., Fliss, A. E., Rao, J., and Caplan, A. J. (1998) Mol. Cell. Biol. 18, 3727–3734
50. Bohen, S. P. (1998) Mol. Cell. Biol. 18, 3330–3339
51. Donze, O., and Picard, D. (1999) Mol. Cell. Biol. 19, 8422–8432
52. Odunuga, O. O., Longshaw, V. M., and Blich, G. L. (2004) Recessions 26, 1058–1068
53. Lee, P., Shabbir, A., Cardozo, C., and Caplan, A. J. (2004) Mol. Cell. Biol. 15, 1785–1792
54. Hu, J., and Poon, R. Y. C. (1997) Trends Cell Biol. 7, 157–161
55. Adkins, B., Hunter, T., and Sethon, B. M. (1982) J. Virol. 43, 448–455
56. Brugge, J. S., Erikson, E., and Erikson, R. L. (1981) J. Biol. Chem. 256, 1429–1429
57. Veldscholte, J., Berrevoets, C. A., Zegers, N. D., van der Kwast, T. H., Grootegoed, L. J., and Mulder, E. (1992) Biochemistry 31, 7422–7430
58. Schulte, T. W., Blagosklonny, M. V., Ingui, C., and Neckers, L. (1995) J. Biol. Chem. 270, 24585–24588
59. Hartson, S. D., and Matts, R. L. (1994) Biochemistry 33, 8912–8920
60. Hartson, S. D., Irwin, A. D., Shao, J., Scroggins, B. T., Volk, L., Myers, P., Lundgren, K., Kan, C. C., and O'Connor, P. M. (2000) Cell 100, 681–692
61. Oe, T., Nakajo, N., Katsuragi, Y., Okazaki, K., and Sagata, N. (2001) J. Biol. Chem. 276, 11648–11656
62. Katsuragi, Y., and Sagata, N. (2004) Mol. Cell. Biol. 24, 1058–1066
63. Scholz, G. M., Hartson, S. D., Cartledge, K., Volk, L., Mats, R. L., and Dunn, A. R. (2001) Cell Growth & Differ. 12, 409–417
64. Ume, S., Hartson, S. D., Chen, J. J., and Mats, R. L. (1997) J. Biol. Chem. 272, 11648–11656