Modular Folding and Evidence for Phosphorylation-induced Stabilization of an hsp90-dependent Kinase*

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The de novo folding of the individual domains of the src family kinase p56<sub>lck</sub> was examined within the context of full-length p56<sub>lck</sub> molecules produced in rabbit reticulocyte lysate containing active chaperone machinery. The catalytic domain required geldanamycin-inhibitable heat shock protein 90 (hsp90) function to achieve its active protease-resistant conformation, but the src homology 2 (SH2) domain acquired phosphopeptide-binding competence independently of hsp90 function. The SH2 domain of hsp90-bound p56<sub>lck</sub> was folded and functional. In addition to the facilitation by hsp90 of kinase biogenesis, a conditional role in maintenance folding could be demonstrated; although wild type p56<sub>lck</sub> molecules with a negative-regulatory C-terminal tyrosine matured to a nearly hsp90-independent state, p56<sub>lck</sub> molecules with a mutated C-terminal tyrosine continued to require hsp90-mediated maintenance. De novo folding could be distinguished from maintenance folding on the basis of proteolytic fingerprints and the effects of different temperatures on folding behavior. Results indicate that during p56<sub>lck</sub> biogenesis, the SH2 domain rapidly folds independently of hsp90 function, followed by the slower hsp90-dependent folding of the catalytic domain and suggest the final stabilization of p56<sub>lck</sub> structure by phosphorylation-mediated interdomain interactions.

Protein domains are discrete units of compact globular structure. They frequently retain structure and function when separated from the whole protein by proteolysis or by protein engineering and are thus often defined as independently folding units. This independent folding of isolated domains may also reflect folding behavior occurring within the context of the whole protein. In vitro studies of protein renaturation support a model in which renaturation of many large, multidomain proteins proceeds through two stages: an initial rapid independent folding of individual protein domains, followed by a slower “domain pairing” stage in which folded domains coalesce to form the active multidomain protein (reviewed in Ref. 1). However, protein folding events occurring within the concentrated environment of the cytosol are facilitated by molecular chaperones, a diverse group of proteins that prevent or reverse deleterious aggregation of protein folding intermediates (reviewed in Ref. 2). Protein folding in vivo is also facilitated by proteins that catalyze potentially rate-limiting steps of in vivo protein folding, the isomerization of peptidyl-prolyl bonds or of disulfide bonds (reviewed in Ref. 3). By these mechanisms, chaperones are thought to both facilitate the de novo folding of newly synthesized proteins and to allow renaturation of partially denatured proteins (reviewed in Refs. 4 and 5). Although our understanding of protein folding in vitro and of chaperone function in vivo have both advanced dramatically in recent years, the influences of molecular chaperones on individual protein folding pathways and processes are not well understood.

The multidomain protein tyrosine kinase p56<sub>lck</sub> provides a unique opportunity to examine the chaperone-mediated folding of individual protein domains within the context of the whole protein. As a typical member of the src family (reviewed in Ref. 6), this nonreceptor protein tyrosine kinase is organized into several discrete domains. Proceeding from the N terminus, these domains include 1) a unique domain that appears to mediate interactions with membranes and integral membrane partner proteins (e.g. CD4; see Ref. 7), 2) an SH3 domain mediating intermolecular or intramolecular interactions (8, 9) interactions with proline-rich type II helices, 3) an SH2 domain mediating intermolecular or intramolecular interactions with specific phosphotyrosine motifs, 4) a highly conserved catalytic domain responsible for phosphotransfer reactions, and 5) a C-terminal tail thought to regulate the kinase via its intramolecular interaction with the SH2 domain.

For p56<sub>lck</sub> and the related kinase viral p60<sub>src</sub> chaperone machinery containing the 90-kDa heat shock protein (hsp90) appears to mediate protein biogenesis (10–14). In addition to de novo folding, hsp90 also appears to supply conditional maintenance of kinase function (10, 15). Because hsp90 function can be specifically inhibited by the benzozquinone anasamycin geldanamycin (16), hsp90-dependent protein folding pathways can be dissected in unfraccionated rabbit reticulocyte lysates (RRL), containing active chaperone machinery (10, 15, 17–19). In the work described here, we utilized this approach to test the hypothesis that chaperone-mediated folding of the full-length p56<sub>lck</sub> molecule occurs in a modular fashion following its de novo folding of individual domains.
novo synthesis. Results indicate a novel chaperone-mediated protein folding pathway in which the SH2 domain rapidly folds independently of hsp90, followed by the slower, hsp90-dependent folding of the catalytic domain. Additionally, we characterized the effects of interactions between the SH2 domain and the kinase regulatory tail and suggest that such interactions may serve as a lynchpin, holding the folded kinase in a stable, hsp90-independent conformation.

EXPERIMENTAL PROCEDURES

Synthesis and Immobilization of Peptides—A high affinity SH2-binding motif derived from the hamster polyoma virus middle T antigen (20) was used as a core sequence for synthesis of four peptides (referred to as mTa peptides) by procedures described previously (21, 22). The four peptides shared the common sequence GGGEQYQEEFI but differed with regard to N-terminal biotinylation and phosphorylation of tyrosine. The peptides were characterized by high performance liquid chromatography and mass spectrometry and were found to be greater than 90% pure. Biotinylated peptides were immobilized on avidin resins (Promega SoftLink Avidin). Nonbiotinylated peptides were used as soluble competitors or were covalently attached to agarose resins (p-nitrophenyl chlorofluorocarbon-activated agarose) using aqueous coupling protocols recommended by the supplier (Sigma).

Solid Phase Peptide Binding Assays—The functional status of p56\(^{lck}\) SH2 domains was determined by assaying the ability of p56\(^{lck}\) folding intermediates (23) to specifically bind to immobilized mTa peptides (24). For these assays, gradient fractions containing either hsp90-bound or free monomeric [\(^{35}\)S]p56\(^{lck}\) were generated as described previously (23), adjusted to contain 1 mM sodium vanadate, and preincubated for 1 h at 4 °C with or without 1.5 μM (2.7 mM) of soluble mTa peptide competitors (lacking biotin). Subsequently, binding reactions were incubated for 1 h on ice with avidin resins to which 10 nmol of the indicated biotinylated mTa peptide had been previously immobilized. Binding reactions were then washed twice with TBS/dt, once with this buffer containing 0.5 M NaCl, and twice more with TBS/dt. Bound materials were analyzed by SDS-PAGE, electrotransfer to PVDF, and autoradiography.

To assess binding of p56\(^{lck}\) and p56\(^{lck}\) folding intermediates directly from RRL translation reactions, reactions were chilled after p56\(^{lck}\) synthesis, treated with 1 mM NaCl, and of the mutant form p56\(^{lck}\) F505 (in which the regulatory tyrosine at position 505 was replaced with phenylalanine (26)) was assessed following translation and de novo folding in Promega TnT reticulocyte lysates. To establish the time required for p56\(^{lck}\) initiation of protein synthesis was arrested. Reactions were further incubated at either 30 °C or 37 °C for 1 h. Alternatively, geldanamycin was added prior to the initiation of p56\(^{lck}\) translation to assess its effects on de novo folding of p56\(^{lck}\). Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute.

Assays of hsp90 Dependence after de Novo Folding—Maturation of wild type p56\(^{lck}\) and of the mutant form p56\(^{lck}\) F505 (in which the regulatory tyrosine at position 505 was replaced with phenylalanine (26)) was assessed following translation and de novo folding in Promega TnT reticulocyte lysates. To establish the time required for p56\(^{lck}\) folding to reach an equilibrium, after which geldanamycin or Me2SO (vehicle control) were added. Reactions were further incubated at either 30 °C or 37 °C for 1 h. Western blotting with 0.5 μg/ml rabbit anti-hsp90 antibodies (PA3-013, Affinity BioReagents). The folding status of soluble and peptide-bound p56\(^{lck}\) molecules was analyzed by mild proteolytic nicking assays as described previously (10, 23) and by the addition of 10 μl of control naive RRL per 250 μl of dilute protease solution prior to its addition to resin-bound protein molecules, respectively. Kinase activities of antibody- or peptide-captured p56\(^{lck}\) molecules were assessed (25), with modifications as described previously (10). Immunoadsorptions of p56\(^{lck}\) were performed using polyclonal antibodies raised in rabbits repeatedly immunized with full-length recombinant p56\(^{lck}\). Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute.

Assays of p56\(^{lck}\) Translation—RRL translation reactions were chilled after p56\(^{lck}\) synthesis, treated with 1 mM NaCl, and incubated for 60 min to allow posttranslational folding to reach an equilibrium, after which geldanamycin or Me2SO (vehicle control) were added. Reactions were further incubated at either 30 °C or 37 °C for 1 h. Alternatively, geldanamycin was added prior to the initiation of p56\(^{lck}\) translation to assess its effects on de novo folding of p56\(^{lck}\). Geldanamycin inhibited binding of [\(^{35}\)S]p56\(^{lck}\) to the control unphosphorylated mTa peptide (Fig. 1B). Specificity of binding was confirmed by preincubation of monomeric [\(^{35}\)S]p56\(^{lck}\) with soluble competitor phosphopeptide or with soluble unphosphorylated mock competitor (Y) prior to incubation with immobilized phosphotyrosine peptide (pY) or with immobilized unphosphorylated peptide (Y). Binding reactions were then washed, eluted, and analyzed by SDS-PAGE, electrotransfer to PVDF, and autoradiography. The full-length lck gene product is indicated (p56\(^{lck}\)). Migrations and molecular masses of standards (kDa) are indicated along the left side of the panel. B, translation reactions were not (lck template, −) or were (lck template, +) programmed for synthesis of [\(^{35}\)S]p56\(^{lck}\). After translation at 30 °C, reactions were chilled, diluted, and incubated with phosphotyrosine peptide resin. Subsequently, resins were washed, eluted, and analyzed by SDS-PAGE, electrotransfer to PVDF, and Western blotting with anti-hsp90 antibodies, hsp90 recovered from binding reactions or applied as standard via an aliquot of RRL is indicated. Migrations and molecular masses of standards (kDa) are indicated along the right side of the panel.

RESULTS

To assess the status of SH2 domains on p56\(^{lck}\) folding intermediates, monomeric and hsp90-bound [\(^{35}\)S]p56\(^{lck}\) populations were produced by de novo synthesis in rabbit reticulocyte lysate translation reactions and separated on glycerol gradients. For each population thus produced, the functional status of its SH2 domain was analyzed by assessing the ability to bind to immobilized phosphotyrosine peptides. Monomeric [\(^{35}\)S]p56\(^{lck}\) showed low levels of nonspecific binding to avidin resins retaining the control unphosphorylated mTa peptide (Fig. 1A). In contrast to this nonspecific binding, resins retaining the phosphotyrosine mTa peptide bound 3-fold more monomeric [\(^{35}\)S]p56\(^{lck}\) than did control resins (Fig. 1A). Specificity of binding was confirmed by preincubation of monomeric [\(^{35}\)S]p56\(^{lck}\) with soluble competitor phosphopeptide; soluble competitor phosphopeptide reduced [\(^{35}\)S]p56\(^{lck}\) binding to background levels (Fig. 1A). In contrast to the phosphorylated soluble competitor peptide (pY), nonphosphorylated competitor peptide (Y) did not inhibit binding of [\(^{35}\)S]p56\(^{lck}\) to phosphopeptide resins (Fig. 1A). Using these criteria for specificity, phosphopeptide resins specifically retained approximately 15–50% of the input mono-
memonic \([^{35}S]p56^{lck}\) isolated from glycerol gradients. Like monomeric \([^{35}S]p56^{lck}\), \([^{35}S]p56^{lck}\) occurring in the p56\(^{lck}\)-hsp90 complex was bound by phosphorylated mTa peptide resins in a specific fashion (Fig. 1A). This binding was qualitatively and quantitatively equivalent to that observed for the monomer similarly produced. These results indicated that the SH2 domain of hsp90-bound p56\(^{lck}\) was folded and functional, or was capable of becoming so during binding assays.

To determine whether hsp90 remained bound to p56\(^{lck}\) during mTa binding, covalently cross-linked mTa phosphopeptide resins were used to adsorb p56\(^{lck}\)-folding intermediates directly from RRL translation reactions. To potentiate the efficiency of mTa binding, these assays utilized a point-mutant of p56\(^{lck}\) in which the regulatory tyrosine at position 505 was mutated to phenylalanine (subsequently called p56\(^{F505}\)). After binding and washing, proteins bound to the mTa peptides were analyzed by SDS-PAGE and Western blotting with anti-hsp90 antiserum. hsp90 showed no affinity for mTa peptide resin in the absence of p56\(^{lck}\) (Fig. 1B, lck template, –). In contrast, hsp90 could be coadsorbed from translation reactions following a brief (30 min at 30 °C) synthesis of p56\(^{F505}\) (Fig. 1B, lck template, +). This specific coadsorption of the hsp90-p56\(^{F505}\) complex was stable under washing with buffers containing up to 2.0 M NaCl (not shown). These results, in agreement with those presented in Fig. 1A, demonstrated that the SH2 domains of hsp90-bound p56\(^{lck}\) molecules were folded and functional.

However, structure/function relationships for the steroid hormone receptor-hsp90 heterocomplex suggested that although hsp90-bound p56\(^{lck}\) folding intermediates might contain folded and functional SH2 domains, hsp90 might nonetheless be required for SH2 domain folding. For steroid hormone receptors, the molybdate-stabilized hsp90-receptor complex is capable of high affinity steroid hormone binding (see, for example, Refs. 27–29). However, hsp90-dependent hormone receptors require hsp90 function to acquire and/or maintain this high affinity hormone-binding conformation (18, 30). To determine whether hsp90 function was necessary for efficient folding of p56\(^{F505}\) SH2 domains within the context of the whole p56\(^{lck}\) molecule, we utilized the hsp90 inhibitor geldanamycin (16), which inhibits hsp90-mediated folding processes by markedly decreasing the \(K_{\text{app}}\) of hsp90 for machinery for ATP (19). Geldanamycin (5 \(\mu\)g/ml) and the drug vehicle Me\(_2\)SO (0.5%, v/v) had no effect on the rates or magnitude of \([^{35}S]p56^{lck}\) synthesis. \(\text{De novo}\) synthesis of \([^{35}S]p56^{lck}\) in the presence of the drug vehicle produced \([^{35}S]p56^{lck}\) molecules with a high competence for mTa binding (Fig. 2, dmso). Binding of \([^{35}S]p56^{lck}\) to unphosphorylated mTa peptide was not observed, nor was binding observed following preincubation with soluble phosphopeptide competitor, indicating that the recovery of \([^{35}S]p56^{lck}\) was specific (Fig. 2). Similarly, synthesis of the \([^{35}S]p56^{lck}\) population in the presence of the hsp90 inhibitor geldanamycin resulted in a population that was competent to specifically bind mTa peptide (Fig. 2, geldanamycin). In this work, as in our previous work (10), geldanamycin-mediated inhibition of kinase folding was not coupled to enhanced degradation of p56\(^{lck}\) by endogenous RRL proteases. These experiments demonstrated that geldanamycin-inhibitable hsp90 function was not necessary for the efficient \(\text{de novo}\) folding and/or stabilization of the p56\(^{lck}\) SH2 domain within the context of the whole p56\(^{lck}\) protein.

Although the folding of the p56\(^{lck}\) SH2 domain was independent of hsp90 function (Fig. 2), we have previously observed that geldanamycin-mediated inhibition of hsp90 function results in the production of p56\(^{lck}\) molecules that are deficient in kinase activity and that have compromised integrity in subsequent protease nicking assays (10). To determine whether SH2-folded and kinase-deficient p56\(^{lck}\) molecules represented two separate populations of p56\(^{lck}\), \([^{35}S]p56^{lck}\) molecules with folded and functional SH2 domains were recovered from control or geldanamycin-treated protein synthesis reactions. p56\(^{F505}\) was the only tyrosine kinase captured by mTa adsorptions from RRL translations (Fig. 3A). Recovery of \([^{35}S]p56^{lck}\) and p56\(^{F505}\) kinase activity by mTa resins was specific, in that its recovery could be prevented by soluble competitor peptide (Fig. 3B, top panel, peptide competitor \(pY\)). Equivalent amounts of \([^{35}S]p56^{lck}\) protein were recovered from

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### Fig. 2. Effects of geldanamycin on \([^{35}S]p56^{lck}\) binding to phosphotyrosine peptide resins. \([^{35}S]p56^{lck}\) was translated in rabbit reticulocyte lysate translation reactions containing either Me\(_2\)SO (dmso) or 5 \(\mu\)g/ml geldanamycin, followed by incubation of protein synthesis reactions in binding buffer containing or lacking the indicated soluble competitor peptides. After prebinding of competitor peptides, reactions were further incubated with the indicated immobilized peptide resins, washed, and eluted by boiling in SDS-PAGE sample buffer prior to analysis by SDS-PAGE, electrotransfer to PVDF, and autoradiography. The full-length lck F505 gene product is indicated (\([^{35}S]p56^{lck}\)). Migrations and molecular masses of standards (kDa) are indicated along the right side of the panel.

#### Fig. 3. Activity of peptide-captured p56\(^{lck}\) produced in the presence of geldanamycin. p56\(^{F505}\) was translated at 30 °C in rabbit reticulocyte lysate reactions, adsorbed to mTa phosphopeptide resins, and assayed for kinase activity. \([^{32}P]p56^{lck}\) and \([^{32}P]\)enolase are indicated and were detected by autoradiography through intervening layers of previously exposed film to quench \(^{35}\)S emission. \([^{35}S]p56^{lck}\) is indicated and was detected by autoradiography after \(^{32}\)P had been allowed to decay for approximately six \(^{32}\)P half-lives. Also indicated is the distortion of electrophoretic migrations of \([^{35}S]p56^{lck}\) proteins caused by decayed enolase present in the lanes (\(\text{en shadow}\)). Migrations and molecular masses of standards (kDa) are indicated along the right side of the panel. A, translation reactions were not (lck template –), or were (lck template +) programmed with template for synthesis of \([^{35}S]p56^{lck}\). After binding and washing, proteins bound to the mTa peptides were analyzed by SDS-PAGE and Western blotting with anti-hsp90 antiserum. hsp90 showed no affinity for mTa peptide resin in the absence of p56\(^{lck}\) (Fig. 1B, lck template, –). In contrast, hsp90 could be coadsorbed from translation reactions following a brief (30 min at 30 °C) synthesis of p56\(^{F505}\) (Fig. 1B, lck template, +). This specific coadsorption of the hsp90-p56\(^{F505}\) complex was stable under washing with buffers containing up to 2.0 M NaCl (not shown). These results, in agreement with those presented in Fig. 1A, demonstrated that the SH2 domains of hsp90-bound p56\(^{lck}\) molecules were folded and functional.

However, structure/function relationships for the steroid hormone receptor-hsp90 heterocomplex suggested that although hsp90-bound p56\(^{lck}\) folding intermediates might contain folded and functional SH2 domains, hsp90 might nonetheless be required for SH2 domain folding. For steroid hormone receptors, the molybdate-stabilized hsp90-receptor complex is capable of high affinity steroid hormone binding (see, for example, Refs. 27–29). However, hsp90-dependent hormone receptors require hsp90 function to acquire and/or maintain this high affinity hormone-binding conformation (18, 30). To determine whether hsp90 function was necessary for efficient folding of p56\(^{F505}\) SH2 domains within the context of the whole p56\(^{lck}\) molecule, we utilized the hsp90 inhibitor geldanamycin (16), which inhibits hsp90-mediated folding processes by markedly decreasing the \(K_{\text{app}}\) of hsp90 for machinery for ATP (19). Geldanamycin (5 \(\mu\)g/ml) and the drug vehicle Me\(_2\)SO (0.5%, v/v) had no effect on the rates or magnitude of \([^{35}S]p56^{lck}\) synthesis. \(\text{De novo}\) synthesis of \([^{35}S]p56^{lck}\) in the presence of the drug vehicle produced \([^{35}S]p56^{lck}\) molecules with a high competence for mTa binding (Fig. 2, dmso). Binding of \([^{35}S]p56^{lck}\) to unphosphorylated mTa peptide was not observed, nor was binding observed following preincubation with soluble phosphopeptide competitor, indicating that the recovery of \([^{35}S]p56^{lck}\) was specific (Fig. 2). Similarly, synthesis of the \([^{35}S]p56^{lck}\) population in the presence of the hsp90 inhibitor geldanamycin resulted in a population that was competent to specifically bind mTa peptide (Fig. 2, geldanamycin). In this work, as in our previous work (10), geldanamycin-mediated inhibition of kinase folding was not coupled to enhanced degradation of p56\(^{lck}\) by endogenous RRL proteases. These experiments demonstrated that geldanamycin-inhibitable hsp90 function was not necessary for the efficient \(\text{de novo}\) folding and/or stabilization of the p56\(^{lck}\) SH2 domain within the context of the whole p56\(^{lck}\) protein.
Methanol- and geldanamycin-treated protein synthesis/folding reactions (Fig. 3B, top panel). However, [35S]p56lck-F505 molecules synthesized in geldanamycin-treated protein synthesis reactions had no detectable kinase activity (Fig. 3B, bottom panel). Inhibition of hsp90 function by geldanamycin resulted in the isolation of a population of p56lck molecules that had folded and functional SH2 domains but lacked kinase activity.

In vitro proteolytic nicking assays of mTa-captured [35S]p56lck were used to further characterize the catalytic domain of mTa-bound [35S]p56lck-F505 molecules produced in the presence of geldanamycin. These assays primarily characterized the folding status of the [35S]p56lck-F505 catalytic domain, because most Met residues present in p56lck occur within the catalytic domain (31, 32). Thus, to determine the folding status of the catalytic domain of mTa-bound [35S]p56lck-F505 was synthesized in translation reactions containing or lacking geldanamycin, [35S]p56lck-F505 was captured by binding to mTa peptide resin, and resin-bonded proteins were subjected to mild native proteolytic nicking. Proteinolytic nicking of peptide-captured molecules synthesized in geldanamycin-treated protein synthesis reactions was performed under nondenaturing conditions and analyzed by SDS-PAGE, electrotransfer to PVDF, and autoradiography. Migration and molecular masses of standards (kDa) are indicated along the right side of the panel.

Within the p56lck C-terminal regulatory tail and its subsequent interaction with the SH2 domain. Before assessing the maintenance of p56lck structure and function by hsp90 machinery, the kinetics of p56lck-F505 folding were assessed. For such assessment, p56lck-F505 was produced in protein synthesis/folding reactions containing active hsp90 machinery. Then, reinitiation of protein synthesis was arrested and the "synchronized" kinase population was subsequently allowed to fold. Incorporation of [35S]Met into newly synthesized p56lck molecules terminated 5–10 min after inhibition of initiation of translation. Similarly, increases in p56lck-F505 kinase activity reached a plateau within 25 min of inhibition of initiation. Thus, the de novo folding of the synchronized p56lck-F505 population reached an equilibrium within 25 min of arrest of initiation of protein synthesis under these conditions.

Using this established time frame, hsp90-mediated maintenance of p56lck-F505 function was assayed by adding geldanamycin well after de novo folding had reached an equilibrium. Nonetheless, late addition of geldanamycin caused p56lck-F505-specific phosphotransferase activity to decline to 36 ± 8% (± 1 S.D.; n = 5) of that seen relative to DMSO controls (e.g. Fig. 5A, F505). In contrast, wild type p56lck molecules were nearly independent of geldanamycin-inhibitable hsp90 function, retaining 88 ± 9% (± 1 S.D.; n = 5) of their phosphotransferase activity following a 1-h incubation at 37 °C in the presence of geldanamycin (e.g. Fig. 5A, wild type). In contrast to the hsp90 dependence shown by p56lck-F505 at 37 °C, neither kinase population showed significant requirements for hsp90-mediated maintenance at the lower temperature of 30 °C (Fig. 5B). Additionally, as wild type kinase molecules acquired hsp90 independence following synthesis and maturation at 37 °C, the
phosphopeptide resin for 3 h, resins were washed and eluted, and p56

Tide resin (chase time 60

ance of the protein synthesis/folding reaction was matured for an addi-

tion to the SDS-PAGE gel to provide a control for antibody specific-

grammed to synthesize wild type [35S]p56

indicated along the

directly to the SDS-PAGE gel to supply a standard for the detection of hsp90 (Fig. 6

Fig. 6. Effects of maturation incubations on intra- and inter-
molecular interactions of p56

A, RRL protein synthesis/folding reactions were (lck template +) or were not (lck template −) pro-
grammed to synthesize wild type [35S]p56

and were incubated for 20

min at 37 °C. Subsequently, initiation of protein synthesis was ar-

rested, polyribosomes were allowed to run off for 7 min, and aliquots of

the synthesis reactions were immediately chilled and incubated with

phosphotyrosine phosphopeptide (chase time 0). Alternatively, the

balance of the protein synthesis/folding reaction was matured for an addi-

tional 60 min at 37 °C prior to chilling and incubation with phosphopep-

tide resin (chase time 60). After each reaction had been incubated with

phosphopeptide resin for 3 h, resins were washed and eluted, and p56

was detected by Western blotting with anti-p56

kDa are indicated.

hsp90-p56

complex became less detectable via communoad-
sorption with anti-p56

inter-
molecular interactions of p56

B

RRL std

p56/lck

RRL std

lck template

chase time

45

66

0

116

97

45

66

0

66

RRL std

hsp90

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Fig. 7. Effects of timing of geldanamycin application on $p56^{lck}$ protease sensitivity. Either drug vehicle (Me$_2$SO, dmso) or geldanamycin was added to RRL protein synthesis/folding reactions prior to synthesis of [$^{35}$S]p$56^{lck}$ at 37°C (de novo). Alternatively, drug additions were performed after p$56^{lck}$ folding had reached an equilibrium in RRL protein synthesis/folding reactions, and the reactions were further incubated for 1 h at 37°C (matured). A and B, after incubation, synthesis/folding reactions were chilled on ice and subjected to mild proteolysis under nondenaturing conditions. Shown is an autoradiogram of [$^{35}$S]p$56^{lck}$ matured in vivo.

Based on these results, we propose that the biogenesis of the multidomain protein tyrosine kinase p$56^{lck}$ occurs via a chaperone-mediated protein folding pathway in which the p$56^{lck}$ SH2 domain folds rapidly, independently of hsp90 function, and function efficiently in context-independent fashions. The proposed model for p$56^{lck}$ folding is consistent with the renaturation pathways for chemically denatured multidomain proteins (reviewed in Ref. 1). The model for p$56^{lck}$ folding is also consistent with postulations that cotranslational folding of individual protein domains may be directed by ribosomal pausing and is consistent with findings that isolated SH2 and SH3 domains fold and function efficiently in context-independent fashions.

In addition to the role of hsp90 in de novo folding, hsp90 is necessary in RRL to maintain the function of p$56^{lck}$ molecules with a single amino acid change in the C-terminal regulatory tail. However, although hsp90 is necessary to maintain the active conformation of p$56^{lck}$F505 in RRL, hsp90-bound p$56^{lck}$ represents a small portion of the total kinase population in vivo.
sensitive, despite the lack of an analogous regulatory phosphorylation event or other posttranslational modifications of p56lck. Additionally, other phosphorylation events may influence its thermal stability. However, preliminary evidence presented here suggests that regulatory phosphorylations may facilitate the production of the mature hsp90-free p56lck molecules detected at the plasma membrane (10). Such phosphorylations may stabilize the catalytic core via enhancing associations between this core and the SH3 and SH2 domains, because deletion of the SH3 and SH2 domains of viral p60releases its association with hsp90 in vivo (for review, see Ref. 34).

However, we do not suggest that hsp90 holds p56lck in a specific static, or “poised,” conformation until regulatory phosphorylation of the kinase triggers physical release from hsp90. Rather, we suggest that hsp90 machinery functions in reiterative cycles of binding and release typical of other chaperones. According to this interpretation, hsp90 releases p56lck molecules irrespective of their phosphorylation status. After release, kinase molecules may experience loss of tertiary structure and may thus require repair by hsp90 machinery, i.e. hsp90 maintains the kinase population via protein refolding. According to this model, the effects of regulatory phosphorylations are to stabilize kinase structure such that this loss of structure does not occur, thus freeing kinase molecules from their dependence on hsp90-mediated refolding and terminating their physical associations with hsp90 machinery.

Consistent with our hypothesis that hsp90 has roles in both the de novo folding and in the conditional maintenance of kinases, newly synthesized p56lckF505 is not equivalent to matured p56lckF505 and with regards to the consequences of hsp90 inhibition. For newly synthesized p56lckF505, geldanamycin-mediated inhibition of hsp90 function produces dramatic hypersensitivity to protease (Fig. 7B, de novo). For “matured” p56lckF505, geldanamycin-mediated inhibition of hsp90 function results in significant loss of kinase specific activity (Fig. 7C), but this loss is not accompanied by detectable changes in protease hypersensitivity (Fig. 7B, matured). Thus, the aberrant structure underlying loss of mature p56lckF505 function is too subtle to be detected by proteolytic nicking assays, Because these proteolytic fingerprints differ depending on whether geldanamycin is applied before or after de novo folding, the tertiary structure of p56lckF505 must develop in an hsp90-dependent fashion during de novo folding. This interpretation is supported by the temperature dependence of the effects of geldanamycin on p56lckF505 “disactivation” versus the temperature-independent effects of geldanamycin on de novo folding (Fig. 5). Furthermore, these results suggest that hsp90 may maintain p56lckF505 structure without reiterating the full de novo folding pathway.

In conclusion, data reported here indicate that hsp90-mediated de novo folding of p56lck proceeds via folding pathways similar to those utilized during in vitro renaturation of purified multidomain proteins in the absence of chaperones. Specifically, p56lck biogenesis appears to occur via a novel chaperone-mediated folding pathway in which the p56lck SH2 domain rapidly folds independently of hsp90 function, followed by the slower, hsp90-dependent folding and maintenance of the catalytic domain prior to the final stabilization of mature protein structure via interdomain interactions. These results suggest that hsp90 machineries may function similarly in other scenarios, folding and refolding substrate proteins prior to the stabilization of their structures by the regulated establishment of intra- or intermolecular interactions between domains. Consistent with this possibility, the physical association of hsp90 with certain transcription factors (46) or kinases (47) can be specific to those forms of these proteins lacking essential partner proteins. Thus, we favor models for the biogenesis of certain regulatory proteins in which pathways of de novo folding and maintenance folding overlap with pathways of regulation.

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Modular Folding and Evidence for Phosphorylation-induced Stabilization of an hsp90-dependent Kinase

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