Hsp90-Mediated Folding of the Lymphoid Cell Kinase p56^{lck}†

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ABSTRACT: Several lines of evidence suggest that members of the 90-kDa family of heat shock proteins (hsp90) may support the folding of various homologues of the *src* kinase family. In this work, we utilized pulse−chase analyses in rabbit reticulocyte lysate to demonstrate that hsp90-bound intermediates existed for the majority of newly synthesized p56^{lck} molecules. The hsp90-binding drug geldanamycin disrupted the association of p56^{lck} with hsp90, prevented the kinase from demonstrating a protease-resistant conformation, and caused decreases in kinase specific activity. Requirements for geldanamycin-inhibitable hsp90 function and physical interactions between hsp90 and p56^{lck} persisted during chase periods. Consistent with the effects observed in rabbit reticulocyte lysate, application of geldanamycin to fibroblasts caused specific reversion of *lck*-mediated transformation concomitant with loss of p56^{lck} activity and protein. However, geldanamycin had no direct effect on purified p56^{lck}. Also consistent with functional linkages between hsp90 and p56^{lck}, physical interactions between these proteins were detected in cytoplasmic, but not membrane, fractions of LSTRA cells. Although hsp90 functions in both the initial *de novo* folding and the reiterative support of p56^{lck} structure in rabbit reticulocyte lysate, the specific occurrence of complexes between hsp90 and p56^{lck} in the cytoplasm of T cells suggests that hsp90 primarily folds nascent molecules of p56^{lck} *in vivo*.

Several lines of evidence indicate that the 90-kDa heat shock proteins (hsp90)[1] facilitate the folding of "substrate" proteins. Hsp90, like other members of the heat-shock-protein superfamily, is essential for cell viability, is expressed both constitutively and during cell stress, and has sequence motifs that are conserved among certain members of the superfamily [reviewed in Craig et al. (1994), Gething and Sambrook (1992), and Jacob and Buchner (1994)]. Like other heat shock proteins, hsp90 prevents the aggregation of proteins *in vitro* (Jacob et al., 1995; Miyata & Yahara, 1992; Wiech et al., 1992) and stimulates the recovery of activity from denatured or improperly folded proteins (Jacob et al., 1995; Miyata & Yahara, 1992; Schumacher et al., 1994; Shaknovich et al., 1992; Shue & Kohtz, 1994; Wiech et al., 1992). The activities or conformations of hsp90-bound substrate proteins change upon dissociation from cytoplasmic hsp90 (Bresnick et al., 1989; Brugge et al., 1983; Courtneidge & Bishop, 1982; Hartson & Matts, 1994; Kost et al., 1989; Sanchez et al., 1985; Ziemiecki, 1986) or from its ER homologue GRP94 (Melnick et al., 1992, 1994). Also consistent with a role for hsp90 in protein folding, genetic evidence indicates that hsp90 supplies an essential positive function for certain signal transduction proteins (Aligue et al., 1994; Carver et al., 1994; Cutforth & Rubin, 1994; Picard et al., 1990; Xu & Lindquist, 1993). However, hsp90 does not appear to play a generic role in folding of all nascent polypeptides (Frydman et al., 1994) but rather appears to act on specific target proteins (Xu & Lindquist, 1993). Thus, hsp90 has been proposed to function as a "signal transduction chaperone", participating in the folding of certain proteins in concert with regulatory stimuli [reviewed in Bohen et al. (1995), Craig et al. (1994), and Rutherford and Zuker (1994)].

Also consistent with a role in protein folding, hsp90 occurs in complexes with substrate polypeptides which are intermediates in the production of active proteins. Prior to activation by hormone, full-length steroid hormone receptors occur in heterocomplexes containing hsp90 and other chaperone machinery [reviewed in Pratt and Welsh (1994)]. After hormone binding, the activated receptors are no longer complexed to hsp90. However, complexes between steroid hormone receptors and hsp90 machinery do not represent static associations, but rather appear to represent a dynamic equilibrium between hsp90-bound and hsp90-free forms of aporeceptors (Smith, 1993). In contrast, the complex formed between viral p60^{src}, hsp90, and the hsp90-cohort p50 does not seem to represent a dynamic equilibrium between hsp90 and the whole cellular population of p60^{src} molecules, but

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[1] Abbreviations: hsp90, heat shock protein 90, denoting members of the family of 90-kDa heat-inducible phosphoproteins; p56^{lck}, the 56-kDa lymphoid cell kinase found in association with the CD4 receptor; p56^{lckF505}, gene product from a *lck* mutation which encodes a phenylalanine at position 505 rather than the wild type regulatory tyrosine; GRP94, the 94-kDa glucose-regulated protein occurring in the endoplasmic reticulum and representing a homolog of hsp90; p60^{src}, nonreceptor tyrosine kinases produced by either cellular or viral homologs of the *src* gene; p50, the 50-kDa phosphoprotein found associated with hsp90 machinery and in complexes with hsp90 and viral p60^{src}; RIPA buffer, radioimmunoprecipitation assay buffer; hsp70, members of the 70-kDa family of heat shock proteins; RRL, rabbit reticulocyte lysate supplemented with buffers, salts, and ATP-regenerating system necessary to support protein synthesis.

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instead seems to be specific for newly synthesized p60^{src} [reviewed in Brugge (1986)]. Similarly, newly synthesized immunoglobulin molecules interact transiently with the hsp90 homologue GRP94 during maturation events occurring in the ER (Melnick et al., 1992, 1994). The mechanisms and relationships behind hsp90's various activities, specificities, and physical associations are not well understood.

In the work presented here, we utilize protein synthesis in rabbit reticulocyte lysate to characterize hsp90-mediated folding of the cellular lymphoid cell kinase p56^{lck}. We provide evidence for two potential roles for hsp90: *de novo* folding of newly synthesized p56^{lck} molecules as well as prolonged maintenance of the structure of p56^{lck} via reiterative equilibrium interactions. We also provide evidence that p56^{lck} requires hsp90 activity *in vivo*.

EXPERIMENTAL PROCEDURES

Cell-Free Modeling of p56^{lck} Maturation. Rabbit reticulocyte translation reactions (Promega) were programmed with template encoding the nonmyristoylated *lck* A2 mutation (Abraham & Veillette, 1990). This nonmyristoylated form of p56^{lck} was utilized to enhance chromatographic separation of p56^{lck} species; equivalent results were observed during chromatography of myristoylated p56^{lck}. For pulse−chase analyses, p56^{lck} synthesis was initiated at 30 °C in the absence of [^{35}S]methionine for 15 min prior to a pulse of 460 μCi/mL [^{35}S]Met (estimated 2 μM Met total in the final protein synthesis mix). After 3 min of radiolabeling at 30 °C, protein synthesis reactions were adjusted to 40 μM unlabeled Met. Half of the reaction was immediately analyzed by size-exclusion HPLC, while the other half was chase−incubated for 1 h further prior to chromatography. Alternatively, radiolabeling was terminated by inhibiting reinitiation of protein synthesis with 10 μM EdeineA and 60 μM aurintricarboxylic acid.

For analysis by HPLC, pulsed or chase−incubated reactions were adjusted to 10 mM sodium molybdate and injected onto a Pharmacia HR 10/30 Superdex 200 column preequilibrated at 4 °C in HPLC buffer containing 25 mM HEPES·NaOH, 25 mM sodium glycerophosphate, 2 mM EDTA, 0.5% Tween20, 10% (w/v) glycerol, 100 mM KCl, and 10 mM sodium molybdate, pH 7.4. Fractions (0.2 mL) eluting from this column were mixed and aliquots were analyzed by SDS−PAGE, autoradiography, and densitometry as previously described (Hartson & Matts, 1994). Immunoadsorptions from individual HPLC fractions were performed as previously described using 8D3 anti-hsp90 antibodies (Hartson & Matts, 1994).

For direct co-immunoadsorption of complexes between hsp90 and p56^{lck}, protein synthesis reactions programmed with *lck* template were assembled to contain or lack 5 μg/mL geldanamycin and specific co-immunoadsorption of hsp90 by anti-p56^{lck} antibodies was detected as previously described (Hartson & Matts, 1994). For assessment of the structure of the *lck* gene product, protein synthesis reactions containing 10 μg/mL geldanamycin were pulse-labeled for 30 min with [^{35}S]methionine, chased by the addition of translation initiation inhibitors and a 4-fold excess of amino acid mix containing unlabeled methionine, and further incubated for an additional 60 min at 30 °C. [^{35}S]p56^{lck} was assayed for protease sensitivity as previously described (Hartson & Matts, 1994).

For assessment of the activity of the *lck* gene product produced in rabbit reticulocyte lysate, the F505 point-mutation of p56^{lck} was utilized to prevent inhibitory phosphorylations of the negative-regulatory tyrosine normally present at position 505. Protein synthesis reactions programmed with *lck* F505 were incubated for 16 min at 30 °C, after which reinitiation of synthesis was arrested by addition of initiation inhibitors. Synthesis was continued for 6 more min to allow runoff of polyribosomes and trapping of p56^{lck} folding intermediates by hsp90. DMSO or geldanamycin was then added to 1% or 10 μg/mL final concentration, respectively, and reactions were incubated for 45 min further to allow maturation of p56^{lck}. To assay for ongoing dependence of p56^{lck} for hsp90, protein was synthesized for 20 min at 37 °C in the absence of initiation inhibitors followed by a 75 min maturation in the presence of 60 μM aurintricarboxylic acid to inhibit reinitiation of protein synthesis. p56^{lck} was immunoadsorbed during the last 45 min of the maturational incubation by resin-bound antibodies directed against the N-terminus of p56^{lck}. After brief centrifugation, the maturational lysate was replaced by fresh lysate with an ATP-regenerating system and containing either 1% DMSO or 1% DMSO/10 μg/mL geldanamycin. These incubations were continued for 45 min at 37 °C, after which the immunopellet was washed and assayed for phosphotransferase activity as described below.

For kinase assays, p56^{lck} was immunoadsorbed, washed with RIPA buffer, and an aliquot (1/10 of total) of the immunoadsorbed material was analyzed by SDS−PAGE and autoradiography to assess immunoadsorption recovery. Using the balance of the immunopellet, kinase activity was determined at 21 °C for 6 min as previously described (Danielian et al., 1989).

Fibroblast Reversion Assays of p56^{lck} Function. Rat 208F fibroblasts transformed by the F505 mutation of the *lck* gene were cultured at 37 °C as described (Hurley et al., 1992). For treatment with geldanamycin, culture medium was replaced with fresh medium containing 60 ng/mL geldanamycin and 0.1% DMSO. Rat 208F fibroblasts transformed by the viral *fos* gene (Curran & Verma, 1984) were treated similarly. Fibroblasts expressing *lck* F505 were lysed in RIPA buffer, and p56^{lck} kinase activity was determined at 21 °C as previously described (Danielian et al., 1989). Kinase assays were analyzed by SDS−PAGE as previously described (Hartson & Matts, 1994) and quantitated by liquid scintillation counting. Direct effects of geldanamycin on p56^{lck} kinase activity were assessed by inclusion of 1.0 μg/mL geldanamycin in kinase assay mixes. Direct effects of genistein were assessed by inclusion of 0.1 mg/mL genistein in kinase assay mixes.

To compare p56^{lck} protein levels in geldanamycin-treated *versus* vehicle-treated 208F/*lck*F505 fibroblasts, cells were treated with geldanamycin or with DMSO alone as described above. After reversion (4 h of treatment), cells were lysed for 45 min at 4 °C in RIPA buffer (2.0 mL of RIPA per 75 cm^2 of cells at approximately 70% confluence). Lysates were clarified at 10 000*g* for 5 min, and the supernates were adjusted slightly to contain equivalent concentrations of total protein. p56^{lck} was detected by direct western blotting or by immunoadsorption of p56^{lck} from lysates followed by western blotting to detect immunoadsorbed p56^{lck}.

Physical Associations between Hsp90 and p56^{lck} in T Cells. The LSTRA T cell line was cultured as described (Voronova

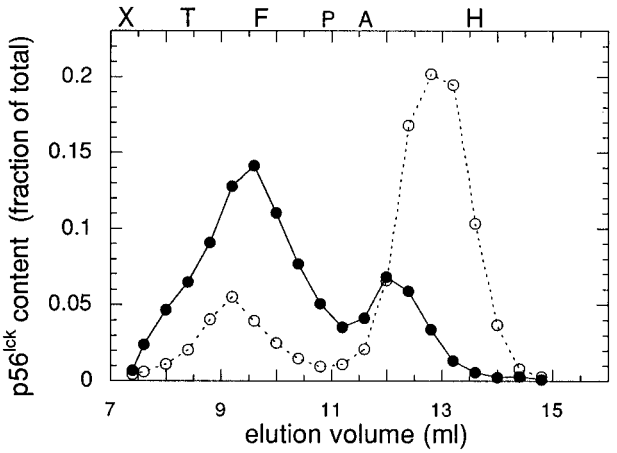

FIGURE 1: Pulse−chase analysis of p56^{lck} maturation in a cell-free system. p56^{lck} was translated in RRL for 15 min prior to a 3-min pulse of [^{35}S]Met. Radiolabeling was then chased with 40 μM unlabeled Met. Half of the reaction was immediately analyzed (●) by size-exclusion HPLC, while the other half of the reaction was chase−incubated (○) for 1 h further prior to chromatography. Aliquots from individual fractions were analyzed by SDS−PAGE, autoradiography, and densitometry of bands representing [^{35}S]p56^{lck}. Individual points represent p56^{lck} eluting at indicated volumes and are expressed as a fraction of the total p56^{lck} detected. Elution volumes of calibration standards are designated across the top of the elution profile (H, hemoglobin, 64 kDa; A, aldolase, 150 kDa; P, pyruvate kinase, 240 kDa; F, ferritin, 440 kDa; T, thyroglobulin, 669 kDa; X, excluded volume of the column).

et al., 1984) at 39 °C/5% CO_2. LSTRA cells were swollen in 25 mM HEPES, 25 mM glycerophosphate, 2 mM EDTA, pH 7.4 (Wartman & Davis, 1994) and lysed by dounce homogenization. Lysates were adjusted to 100 mM KCl, clarified by centrifugation at 1500*g*, and separated into cytosolic and membrane fractions by centrifugation for 30 min at 100 000*g*. Cellular membranes were resuspended in lysis buffer containing 1% Triton X-100, 0.25% deoxycholate and incubated with rocking at 4 °C for 30 min. The cytosol fraction was adjusted to contain 1% Triton X-100 and 0.25% deoxycholate and was similarly incubated. Membrane and cytosol fractions were further clarified by centrifugation for 30 min at 100 000*g* and subjected to immunoadsorption with polyclonal anti-p56^{lck} (Amrein et al., 1992) bound to Protein G-Sepharose. Anti-p56^{lck} immunoresins were incubated with cell fractions for 2 h at 4 °C, washed as previously described (Hartson & Matts, 1994), and washed finally with 10 mM Tris·HCl (pH 7.4), 50 mM NaCl. Because detection of hsp90 in anti-p56^{lck} immunoadsorption pellets was compromised by reactivity between the adsorbing antibody and the secondary antibody used for western blot detection, two properties of the interaction between hsp90 and viral p60^{src} were utilized to elute hsp90 from the adsorbed complex while eluting minimal amounts of the adsorbing antibodies from resin. These properties were the reported instability of the complex in the absence of salt (Brugge, 1986) and at elevated temperatures (Hutchison et al., 1992a,b). Thus, immunoresins were eluted at 30 °C for 45 min in 5 resin volumes of 10 mM Tris·HCl (pH 7.4). Eluted material was analyzed by western blotting with 4322 anti-hsp90/hsp70 antiserum (Ehrhart et al., 1988) as previously described (Hartson & Matts, 1994). To isolate hsp90 by direct immunoadsorption, LSTRA cytosol lysate was diluted with 2 volumes of water and immunoadsorbed with monoclonal anti-hsp90 as previously described (Hartson & Matts, 1994).

RESULTS

Nature of Interactions between Hsp90 and p56^{lck} in Rabbit Reticulocyte Lysate. Protein synthesis in rabbit reticulocyte lysate (RRL) has been used by many groups to provide a very high-fidelity model for chaperone-mediated folding of protein molecules (Frydman et al., 1994; Jacob et al., 1995; Mattingly et al., 1993; Schumacher et al., 1994). We have previously used this system to show that translation reactions programmed with *lck* template produced two forms of [^{35}S]-p56^{lck}, a free monomer that co-sedimented with hemoglobin and a discrete fast-sedimenting population bound to hsp90 (Hartson & Matts, 1994). In work presented here, we utilized a similar approach to determine whether the distribution of p56^{lck} among these species represented a nonproductive "dead-end" pathway, a productive unidirectional pathway, or a dynamic equilibrium. For this determination, protein synthesis reactions programmed with *lck* message were subjected to pulse or pulse−chase radiolabeling and analyzed by size exclusion HPLC.

Immediately following pulse labeling and gel filtration, two discrete peaks of newly synthesized p56^{lck} molecules were observed (Figure 1). Approximately 75% of newly synthesized p56^{lck} molecules occurred in large-radius complexes eluting at 9.4 mL, slightly before ferritin (ferritin M_r = 440 kDa). p56^{lck} molecules occurring in this complex could be co-immunoadsorbed by anti-hsp90 antibodies. In addition to this population, a second peak of newly synthesized p56^{lck} molecules was observed eluting at 12.0 mL, slightly after alcohol dehydrogenase (alcohol dehydrogenase M_r = 150 kDa). For this second peak, immunoadsorption with anti-hsp90 antibodies did not co-adsorb p56^{lck}. Importantly, p56^{lck} was not observed eluting in the column void volume nor did radiolabel accumulate in the guard column, indicating that high-weight aggregates did not form following *de novo* synthesis in RRL.

When radiolabeling was chased by incubation at 30 °C in the presence of excess Met, approximately 75% of the p56^{lck} population was recovered in a peak eluting at 12.8 mL (Figure 1), slightly before hemoglobin (hemoglobin M_r = 64 kDa). This chased peak was distinct from the peak eluting at 12.0 mL that was observed in newly synthesized p56^{lck} populations. This chased peak contained p56^{lck} molecules that were not co-immunoadsorbed by anti-hsp90 antibodies. In addition to this chased peak, approximately 25% of chased p56^{lck} molecules occurred in the large-radius hsp90-bound complexes eluting at 9.4 mL. p56^{lck} was similarly distributed between peaks eluting at these positions when radiolabeling was chased by the arrest of reinitiation of protein synthesis.

Geldanamycin Inhibition of Hsp90-Dependent p56^{lck} Folding. To gain a better understanding of the function of hsp90 with regards p56^{lck}, we utilized the hsp90-binding drug geldanamycin. Geldanamycin disrupts normal interactions between hsp90 chaperone machinery and many hsp90-dependent proteins, presumably via inhibition of one or more activities of hsp90 [e.g., see Whitesell et al. (1994)]. Additionally, geldanamycin inhibits the hsp90-dependent renaturation of heat-denatured firefly luciferase (Thulasiraman & Matts, 1996). Thus, we characterized the effects of geldanamycin on three properties of p56^{lck} synthesized and matured in RRL: associations of p56^{lck} with hsp90; resistance of p56^{lck} to mild proteolytic nicking; and p56^{lck} specific activity.

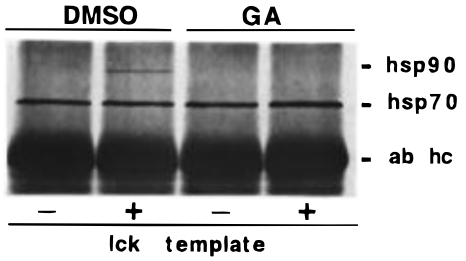

FIGURE 2: Effect of geldanamycin on hsp90:p56^{lck} complex in a cell-free system. Geldanamycin (GA) or DMSO (DMSO) was added to *in vitro* translation reactions that were (*lck*+) or were not (*lck*−) programmed with *lck* template. Reactions were then incubated at 30 °C for 45 min, chilled on ice, and immunoadsorbed with rabbit anti-p56^{lck} antibodies. Immunoadsorptions were analyzed by SDS−PAGE and western blotting with rabbit anti-(hsp90/hsp70) antisera. Hsp90, hsp70, and the heavy chain of the immunoadsorbing antibody (ab hc) are indicated.

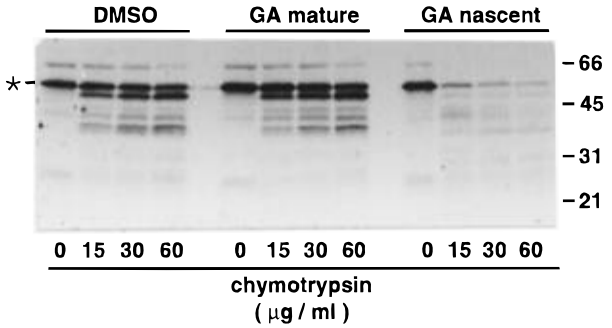

FIGURE 3: Effect of geldanamycin on the structure of p56^{lck} produced in a cell-free system. [^{35}S]p56^{lck} was produced in *in vitro* translation reactions containing either DMSO (DMSO) or geldanamycin added either after (GA mature) or before (GA nascent) synthesis of p56^{lck}. Translation reactions were chilled on ice and treated with the indicated amounts of chymotrypsin for 6 min. Proteolyzed samples were analyzed by SDS−PAGE and autoradiography. The full-length *lck* translation product is indicated (∗). Migrations and molecular masses of standards (kDa) are indicated along the right side of the panel.

To determine if geldanamycin affected the association of p56^{lck} with hsp90, we utilized co-immunoadsorption of complexes between hsp90 and p56^{lck}. This approach was selected to simplify analysis of complex populations of p56^{lck} molecules. As reported previously (Hartson & Matts, 1994), association of hsp90 with p56^{lck} could be detected via specific co-adsorption of hsp90 by antibodies directed against the C-terminus of p56^{lck} (Figure 2). The drug vehicle, DMSO, did not disrupt this association of p56^{lck} with hsp90 (Figure 2). However, following synthesis of p56^{lck} in the presence of geldanamycin, the association of p56^{lck} with hsp90 was not detected (Figure 2). Geldanamycin did not affect rates nor levels of p56^{lck} synthesis nor did geldanamycin affect the efficiency of anti-p56^{lck} immunoadsorption. Thus, geldanamycin disrupted the interaction of p56^{lck} with chaperone machinery containing hsp90.

To determine if this disruption correlated with any effects on the structure of p56^{lck}, proteolytic nicking assays of [^{35}S]-p56^{lck} structure were utilized. These assays primarily characterized the p56^{lck} kinase domain, since most [^{35}S]-methionine residues occurred within this domain (Marth et al., 1985). Translation and maturation of p56^{lck} in the presence of geldanamycin resulted in p56^{lck} molecules that were very hypersensitive to proteolysis relative to those produced in the absence of this compound (Figure 3). Exaggerated proteolysis did not result from activation of an endogenous protease such as the proteosome (Tsubuki et al., 1994), since no proteolytic breakdown of p56^{lck} was observed in the absence of added protease (Figure 3). These results implied that hsp90 was necessary for p56^{lck} to acquire or maintain stable tertiary structure.

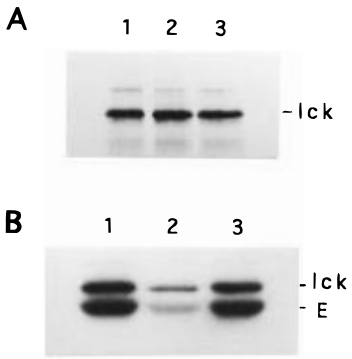

FIGURE 4: Effect of geldanamycin on maturation of p56^{lckF505} activity in a cell-free system. p56^{lckF505} was synthesized in translation reactions lacking geldanamycin followed by the termination of protein synthesis with initiation inhibitors. Geldanamycin or drug vehicle was added to these reactions, and the kinase was allowed to mature at 30 °C. Additionally, geldanamycin was added to p56^{lckF505} matured in the absence of geldanamycin and incubated an additional 5 min. Subsequently, p56^{lckF505} was isolated by immunoadsorption, an aliquot of the immunoadsorption was analyzed by SDS−PAGE and autoradiography, and kinase activity was assayed using the balance of the immunopellet. Bands representing isotopically labeled p56^{lckF505} (*lck*) and [^{32}P]enolase (E) are indicated. Panel A: lane 1, [^{35}S]p56^{lck} immunoadsorbed from maturation reactions treated with DMSO; lane 2, [^{35}S]p56^{lckF505} immunoadsorbed from maturation reactions treated with geldanamycin; lane 3, [^{35}S]p56^{lckF505} immunoadsorbed from maturation reactions to which geldanamycin was added after p56^{lckF505} maturation. Panel B: lane 1, kinase activity immunoadsorbed from maturation reactions treated with DMSO; lane 2, kinase activity immunoadsorbed from maturation reactions treated with geldanamycin; lane 3, kinase activity immunoadsorbed from maturation reactions to which geldanamycin was added after p56^{lckF505} maturation.

Since hsp90 was observed to bind the majority of newly synthesized p56^{lck} molecules within 3 min of their translation (Figure 1), we wished to test the hypothesis that once hsp90 had "trapped" p56^{lck} folding intermediates, geldanamycin would no longer inhibit their maturation. To test this hypothesis, p56^{lck} was briefly synthesized in untreated RRL and subsequently incubated in geldanamycin-treated RRL. These assays utilized the F505 mutant of p56^{lck} (p56^{lckF505}) to allow interpretation of kinase assays without considering the effects of phosphorylation of the negative regulatory tyrosine normally present at this position. Following such incubation, p56^{lckF505} was immunoadsorbed with anti-p56^{lck} antibodies and its kinase activity assayed. These kinase assays were specific for p56^{lck} activity, as no phosphorylation of enolase was observed in immunopellets from mock translations programmed with water. Immunoadsorbed p56^{lckF505} matured in the presence of geldanamycin catalyzed less *in vitro* autophosphorylation and less phosphorylation of the exogenous substrate enolase than did p56^{lckF505} matured in the presence of the drug vehicle, DMSO (Figure 4). Addition of geldanamycin for brief periods (5 min) after p56^{lckF505} maturation had no detectable effects on kinase activity (Figure 4B, lane 3). The specific activity of the p56^{lckF505} produced in the absence and presence of geldanamycin was quantitated via densitometry of an appropriately exposed autoradiogram and by liquid scintillation counting

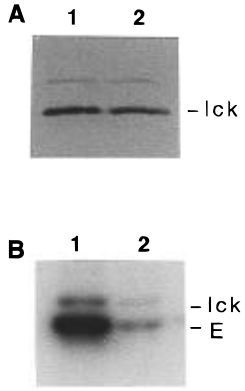

FIGURE 5: Effect of geldanamycin on maintenance of p56^{lckF505} activity in a cell-free system. p56^{lckF505} was synthesized in translation reactions lacking geldanamycin followed by the termination of protein synthesis with initiation inhibitors. The kinase was allowed to mature for 75 min at 37 °C. After maturation, the original lysate containing immunoadsorbed p56^{lckF505} was replaced by fresh lysate containing either 1% DMSO or 1% DMSO/10 μg/mL geldanamycin. Incubations were continued for 45 min at 37 °C, after which an aliquot of the immunoadsorption was analyzed by SDS−PAGE and autoradiography. Kinase activity was assayed using the balance of the immunopellet. Bands representing isotopically labeled p56^{lckF505} (*lck*) and [^{32}P]enolase (E) are indicated. Panel A: lane 1, [^{35}S]p56^{lckF505} incubated in lysate treated with DMSO; lane 2, [^{35}S]-p56^{lckF505} incubated with lysate treated with geldanamycin. Panel B: lane 1, kinase activity of p56^{lckF505} incubated in RRL treated with DMSO; lane 2, kinase activity of p56^{lckF505} incubated in RRL treated with geldanamycin.

of excised bands of [^{35}S]p56^{lckF505} and [^{32}P]enolase. This quantification showed that the specific activity of p56^{lckF505} matured at 30 °C in geldanamycin-treated RRL was 15%−20% of that matured in the absence of geldanamycin. These results indicated that the thesis hypothesis was false; prior trapping of p56^{lckF505} folding intermediates did not protect them from the deleterious effects of hsp90 inhibition.

Because prior trapping of p56^{lckF505} in complexes with hsp90 chaperone machinery did not prevent the deleterious effects of hsp90 inhibition, we hypothesized that p56^{lckF505} molecules produced in RRL required continued reiterative support from hsp90 chaperone machinery. To test this hypothesis, we utilized geldanamycin inhibition of hsp90 to determine if "mature" molecules of p56^{lckF505} produced in RRL still required hsp90 support. For these determinations, the kinase was produced and matured in the absence of geldanamycin, followed by transfer of the kinase to geldanamycin-treated RRL. These studies were performed at 37 °C to reflect protein folding at physiologically relevant temperatures. Even after prior "maturation" of p56^{lckF505}, geldanamycin-mediated inhibition of hsp90 resulted in a 95% decrease in p56^{lckF505} specific activity (Figure 5). These results indicated that p56^{lckF505} produced in RRL required continuous support of folding from geldanamycin-inhibitable hsp90 machinery.

Functional Interactions between Hsp90 and p56^{lck} in Fibroblasts. To determine if results from RRL modeling of functional interactions of hsp90 with p56^{lck} or with p56^{lckF505} were applicable to kinase maturation and function *in vivo*, functional interactions between hsp90 and p56^{lckF505} were examined in rat 208F fibroblasts transformed by this kinase. In fibroblasts expressing p56^{lckF505}, uncompromised p56^{lckF505} kinase activity causes rounded or spindle-shaped cell morphologies (Adler & Sefton, 1992; Amrein & Sefton, 1988; Marth et al., 1988). However, if p56^{lckF505} function is

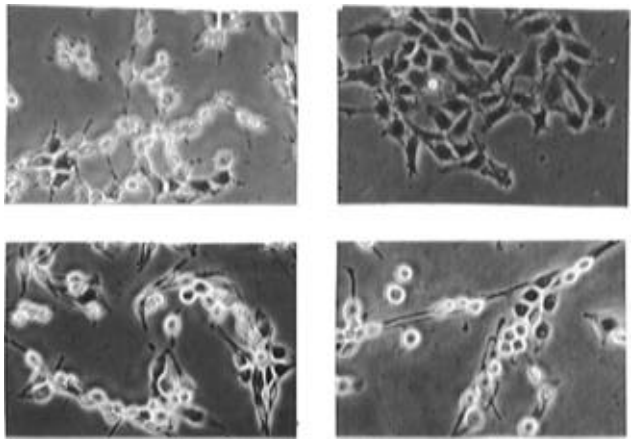

FIGURE 6: Effect of geldanamycin on p56^{lck}-mediated transformation. Rat fibroblasts transformed by the F505 mutation of the *lck* gene (top-left and top-right panels) or the viral *fos* gene (bottom-left and bottom-right panels) were treated with 0.1% DMSO (top-left and bottom-left panels) or with 60 ng/mL geldanamycin/0.1% DMSO (top-right and bottom-right panels). Cultures were incubated under these conditions for 4 h at 37 °C and photographed under phase-contrast microscopy.

compromised, the more typical flattened fibroblast morphologies are observed (Abraham & Veillette, 1990; Hurley et al., 1992). Thus, fibroblast reversion assays of p56^{lckF505} function were utilized to examine the effects of geldanamycin on p56^{lckF505} function *in vivo*. The geldanamycin vehicle, DMSO, had no effect on the rounded morphologies characteristic of transformation mediated by p56^{lckF505} (Figure 6, top left). However, in the presence of 60 ng/mL geldanamycin, these cells rapidly reverted to a flattened, less cytoplasmically dense morphology, indicating a reversion of p56^{lckF505}-mediated transformation (Figure 6, top right). Cell viability was not affected by short-term treatment with geldanamycin.

To determine if geldanamycin-mediated reversion of transformation was due to a general effect on cell metabolism or on the transformation process, geldanamycin was applied to 208F cells transformed by an oncogene unrelated to the *src* family kinases, the viral homologue of the *fos* transcription factor. In contrast to p56^{lckF505}-mediated transformation (Figure 6, top left and right), geldanamycin (60−1000 ng/mL) had no effects on the morphology of *fos*-transformed fibroblasts (Figure 6, bottom left and right). Thus, reversion of fibroblast transformation by geldanamycin was not due to nonspecific effects on cellular metabolism or on the transformation process but was instead specific for some aspect of *lck*-mediated transformation.

Specific reversion of *lck*-mediated fibroblast transformation suggested that geldanamycin caused loss of p56^{lckF505} function *in vivo*. To test this interpretation, the amount of p56^{lckF505} kinase activity present in lysates of geldanamycin-treated fibroblasts was determined via immunoadsorption with anti-p56^{lck} antibodies followed by *in vitro* assays of recovered kinase activity. These kinase assays were specific for p56^{lck}, as evidenced by failure to detect [^{32}P]products in assays of peptide-neutralized immunoadsorptions. Treatment of 208F/*lck* F505 fibroblasts with 60 ng/mL geldanamycin resulted in the loss of 60% of the p56^{lckF505} activity detected in cell lysates (Figure 7A) concomitant with reversion of transformation (Figure 6). This result was consistent with the hypothesis that geldanamycin caused the loss of p56^{lckF505} function *in vivo*.

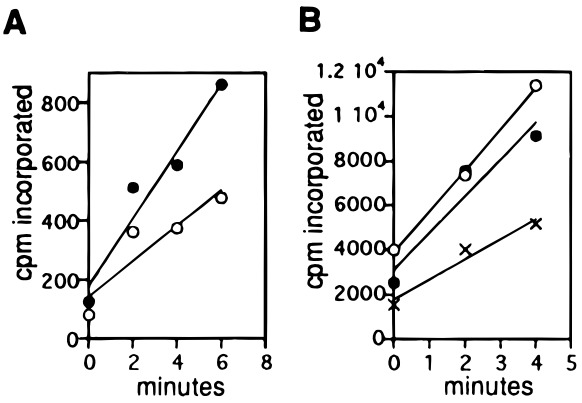

FIGURE 7: Effects of geldanamycin of p56^{lck} kinase activity *in vivo* or *in vitro*. (A) Rat 208F fibroblasts expressing *lck* F505 were treated with DMSO (●) or with 60 ng/mL geldanamycin (○) 4 h prior to lysis and immunoadsorption with anti-p56^{lck} antibodies. Aliquots of immunoadsorptions were incubated for the indicated times in kinase reactions with [γ-^{32}P]ATP. Autophosphorylation of p56^{lck} was analyzed by SDS−PAGE and liquid scintillation counting of excised bands. (B) p56^{lck} was isolated from LSTRA cells by immunoadsorption and assayed *in vitro* with the addition of 0.1% DMSO (●), 1.0 μg/mL geldanamycin (○), or 0.1 mg/mL genistein (×) directly to kinase reactions. Autophosphorylation of p56^{lck} was analyzed by SDS−PAGE and liquid scintillation counting of excised bands.

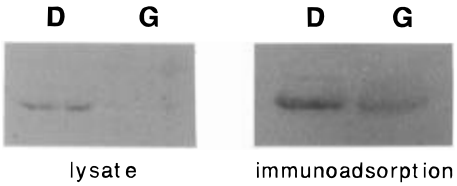

FIGURE 8: Comparison of p56^{lck} levels in vehicle-treated *versus* geldanamycin-treated fibroblasts. Cells were treated either with DMSO (D) or with 60 ng/mL geldanamycin dissolved in DMSO (G) for 4 h. RIPA lysates were prepared and subjected to SDS−PAGE and western blotting with anti-p56^{lck} antibodies (lysate). Alternatively, p56^{lck} was concentrated from RIPA lysates by immunoadsorption with anti-p56^{lck} antibodies prior to SDS−PAGE and western blotting with anti-p56^{lck} antibodies (immunoadsorption).

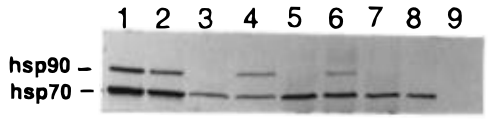

FIGURE 9: Immunoadsorption of hsp90 from LSTRA cell fractions. LSTRA cells were lysed, fractionated into cytosol and membrane components, and subjected to immunoadsorption as described in Experimental Procedures. Aliquots of LSTRA cytosol (lane 1) or membrane (lane 2) fractions were loaded directly to an SDS−PAGE gel. Immunoadsorptions of cytosol (lanes 3−6) or membrane (lanes 7 and 8) fractions utilized control nonimmune antibodies (lanes 3, 5, and 7), antibodies against hsp90 (lane 4), or antibodies against recombinant p56^{lck} (lanes 6 and 8). Immunoadsorption reactions and naive anti-p56^{lck} antibodies (lane 9) were eluted as described in Experimental Procedures and analyzed by SDS−PAGE and western blotting with anti-hsp90/hsp70 antisera. Hsp90 and hsp70 are indicated.

To determine if this loss represented direct inhibition of the kinase by geldanamycin, p56$^{lck F505}$ isolated from untreated fibroblasts and p56^{lck} isolated from untreated LSTRA cells were assayed directly in the presence of 1.0 μg/mL geldanamycin, a concentration approximately 17-fold greater than the effective *in vivo* dose. Direct inhibition of autophosphorylation or phosphorylation of enolase was observed neither in kinetic assays of p56^{lck} from LSTRA cells (Figure 7B) nor in single-point kinase assays of p56$^{lck F505}$ isolated from untreated 208F/*lck* F505 cells (not shown). The failure to detect direct inhibition by geldanamycin did not reflect limitations of the assay conditions, since the unrelated kinase inhibitor genistein inhibited p56^{lck} kinase activity *in vitro* by 54% under these conditions (Figure 7B). Thus, geldanamycin did not directly inhibit p56^{lck} activity *in vitro* but rather geldanamycin reduced p56^{lck} activity and function through an indirect mechanism.

To determine if losses in p56$^{lck F505}$ kinase activity from fibroblasts were correlated with loss of p56$^{lck F505}$ protein, lysates from cells treated with 60 ng/mL geldanamycin were western blotted with monoclonal mouse antibodies directed against p56^{lck}. Additionally, p56$^{lck F505}$ in cell lysates was concentrated by immunoadsorption with rabbit anti-p56^{lck} antibodies and detected by western blotting with mouse anti-p56^{lck} antibodies. Both approaches indicated that during geldanamycin-mediated reversion of *lck*-transformed fibroblasts, the amounts of p56$^{lck F505}$ protein present in cell lysates was reduced (Figure 8). To estimate the magnitude of this reduction, bands representing p56$^{lck F505}$ recovered by immunoadsorption with rabbit antibodies were analyzed by densitometry. This analysis suggested that geldanamycin treatment reduced p56$^{lck F505}$ protein levels to approximately 50% of those observed in the control cells treated with drug vehicle.

Physical Interactions between Hsp90 and p56^{lck} in T Cells. Modeling in RRL and in rat fibroblasts indicated that hsp90 function could be essential to that of p56^{lck}. To determine if these analyses were applicable to events occurring in T cells, we attempted to demonstrate a physical interaction between hsp90 and p56^{lck} in LSTRA cells, a T cell lymphoma. These cells express wild-type p56^{lck} at elevated levels (Marth et al., 1985). Anti-hsp90/hsp70 4322 antiserum detected hsp90 and hsp70 in both the cytosol and membrane fractions from LSTRA cell lysates (Figure 9, lanes 1 and 2, respectively). The identification of hsp90 was confirmed by specific immunoadsorption of this protein by anti-hsp90 monoclonal antibodies (Figure 9, lanes 3 and 4). When LSTRA cytosols were immunoadsorbed with anti-p56^{lck} antiserum, hsp90 was detected in material eluted (see Methods) from the anti-p56^{lck} immunoresin (Figure 9, lanes 5 and 6). In contrast, similar co-adsorption of hsp90 was not detected from membrane fractions from these cells (Figure 9, lanes 7 and 8). The specific occurrence of this complex in cytosolic fractions, but not membrane fractions, demonstrated that co-adsorption did not represent spurious cross-reactivity between anti-p56^{lck} antiserum and hsp90. Additionally, although nonspecific binding of hsp70 to immunoresins was apparent in most lanes, nonspecific binding between hsp90 and nonimmune antibodies was not observed (Figure 9; lanes 3, 5, and 7) nor was hsp90 detected in the resin-bound immune antiserum prior to exposure to LSTRA lysates (Figure 9, lane 9). Hsp90 eluted from anti-p56^{lck} immunoadsorptions could be readsorbed by 8D3 monoclonal anti-hsp90 antibody. Additionally, hsp90 could be co-adsorbed during anti-p56^{lck} immunoadsorptions of LSTRA cell lysates prepared in the absence of detergent. The specific co-adsorption of hsp90 by anti-p56^{lck} antibodies demonstrated that complexes containing both p56^{lck} and hsp90 existed in cytosolic fractions from LSTRA cells. On the basis of the amount of hsp90 recovered from anti-p56^{lck} immunoadsorptions of LSTRA cytosol and the observation that membrane-bound p56^{lck} was not associated with hsp90,

hsp90-bound p56^{lck} represents a small fraction of the total cellular population of p56^{lck} molecules.

DISCUSSION

Available data indicate that geldanamycin is a powerful tool that can be used to dissect certain hsp90-dependent processes. Neither geldanamycin nor the related compound herbimycin A directly binds *src*-family kinases (Whitesell et al., 1994). Instead, geldanamycin binds to hsp90 (Whitesell et al., 1994) and disrupts normal interactions between hsp90 machinery and a variety of hsp90 "substrates", including viral p60^{src} (Whitesell et al., 1994), p185^{erb-B2} (Chavany et al., 1996), the progesterone receptor (Johnson & Toft, 1995; Smith et al., 1995), the glucocorticoid receptor (Whitesell et al., 1995), and the raf kinase (Schulte et al., 1995). This disruption correlates with *in vivo* loss of function and accelerated turnover for hsp90-associated signal transduction proteins. Thus, an accumulating body of evidence indicates that geldanamycin inhibits hsp90's support of certain signal transduction proteins. This putative mechanism contrasts sharply with traditional models suggesting that benzoquinonoid ansamycins inhibit tyrosine kinases directly via covalent derivatization of vulnerable cysteines (Fukazawa et al., 1994). However, direct derivatization of tyrosine kinases has not been demonstrated under physiologically relevant conditions. Additionally, the biologically active benzoquinonoid ansamycins geldanamycin[2] and 19-allylaminoherbimycin A (Makishima et al., 1995) are not reactive to thiol compounds.

Our results indicate that in the rabbit reticulocyte model system (RRL), geldanamycin-inhibitable hsp90 activity is necessary for the proper folding of the lymphoid cell kinase, p56^{lck}. p56^{lck} interacts with hsp90 following *de novo* synthesis in RRL, and this interaction correlates with altered kinase structure and activity (Hartson & Matts, 1994). Disruption of the interaction between hsp90 and p56^{lck} by geldanamycin (Figure 2) results in the production of p56^{lck} molecules that are deficient in kinase specific activity (Figures 4 and 5) and whose kinase domains are very hypersensitive to mild proteolytic nicking (Figure 3). These nicking assays measure the ability of substrate to deform locally and occupy the catalytic cleft of the protease without disturbing the stable tertiary structure of the substrate (Hubbard et al., 1994). Thus, the dramatic hypersensitivity to protease indicates that p56^{lck} molecules synthesized in the presence of geldanamycin are deficient in the stable tertiary structure necessary to protect them from proteolysis, i.e., they are misfolded or incompletely folded. This result indicates that one function of hsp90 machinery is to support the folding of substrate proteins.

Geldanamycin and other benzoquinonoid ansamycins probably cause similar disruptions of hsp90-dependent folding of signal transduction proteins *in vivo*, leading to the frequently reported loss of function and destabilization of hsp90-associated proteins by these drugs. Consistent with geldanamycin-mediated inhibition of hsp90-dependent folding of p56^{lck} in RRL, geldanamycin inhibits p56^{lck} function and activity when p56$^{lck\text{F505}}$ is expressed in rat fibroblasts. Geldanamycin causes specific reversion of *lck*-mediated transformation (Figure 6) concomitant with loss of p56$^{lck\text{F505}}$ activity (Figure 7A) and protein (Figure 8). However, these losses in kinase activity are unlikely to represent direct inhibition of p56^{lck} (Figure 7B). Instead, these observations are consistent with the disruption of hsp90-dependent folding of p56$^{lck\text{F505}}$ followed by clearing of misfolded p56$^{lck\text{F505}}$ molecules from the cell by proteolytic turnover.

Consistent with functional linkages between hsp90 and p56^{lck} in model systems, physical interactions between hsp90 and p56^{lck} can be detected in cytoplasmic lysates of LSTRA cells (Figure 9). The specific occurrence of this complex in only the cytosolic fraction has three implications: (1) the complex is unlikely to represent an *in vitro* artifact due to binding of p56^{lck} to hsp90 during the preparation of lysate, since such an artifact would be unlikely to be fraction specific; (2) hsp90 interacts with cytoplasmic p56^{lck} *in vivo*; and (3) hsp90 does not interact with active membrane-bound p56^{lck}. We believe that two factors contribute to previous failures to detect physical interactions between hsp90 and cellular *src* family kinases: (1) the low stoichiometry of such interactions at any given time; and (2) potential destabilization of these complexes in the strong ionic detergents typically used to lyse adherent cells (Hartson & Matts, 1994). Additionally, failure to detect genetic interactions between hsp90 and cellular p60^{src} in yeast (Xu & Lindquist, 1993) may reflect the weak phenotype of this kinase in yeast.

In the RRL cell-free model of protein biogenesis, hsp90 can play two complementary roles in supporting the folding of p56^{lck}. One of these roles is to the facilitate the *de novo* folding of newly synthesized molecules of p56^{lck}. Pulse−chase analyses demonstrate that most, if not all, newly synthesized p56^{lck} molecules proceed through an hsp90-bound intermediate to produce free monomer (Figure 1). Similar flow of newly synthesized proteins through hsp90-bound intermediates has been described for the *in vivo* maturation pathways of viral p60^{src} (Brugge et al., 1983; Courtneidge & Bishop, 1982), other oncogenic tyrosine kinases (Ziemiecki, 1986), and the GRP94-dependent folding of immunoglobulin chains in the endoplasmic reticulum (Melnick et al., 1992, 1994). However, in addition to folding newly synthesized p56^{lck} molecules, hsp90 can play a second role in support of p56$^{lck\text{F505}}$ folding in RRL, namely, the continued maintenance of kinase structure. Even after prolonged "maturation" of p56$^{lck\text{F505}}$ in RRL, inhibition of hsp90 machinery can still result in dramatic losses in p56$^{lck\text{F505}}$ specific activity (Figure 5). Thus, the p56^{lck} population can have a prolonged requirement for reiterative support from hsp90 chaperone machinery. This continuing need for support from hsp90 suggests that the distribution between hsp90-bound and monomeric forms of the kinase that we observed during chase periods may be a dynamic equilibrium, resembling that previously described for hsp90's association with the progesterone receptor (Smith, 1993).

Modeling in RRL demonstrates two potential roles for hsp90 in mediating p56^{lck} folding. Our data support the existence of one of these roles in T cells, namely, the support of *de novo* folding. Three properties of the physical interaction between hsp90 and p56^{lck} suggest that hsp90 machinery interacts with newly synthesized p56^{lck} in T cells. (1) Most newly synthesized p56^{lck} molecules associate with hsp90 within 3 min after their synthesis in the high-fidelity RRL model (Figure 1), suggesting that newly synthesized p56^{lck} molecules would require similar support from hsp90 chaperone machinery in T cells. (2) Complexes between

[2] L. Whitesell, personal communication.

hsp90 and p56^{lck} are specific to cytoplasmic fractions of LSTRA cells (Figure 9), as are similar complexes between hsp90 and newly synthesized viral p60^{src} (Brugge et al., 1983; Courtneidge & Bishop, 1982). (3) Hsp90-bound p56^{lck} appears to represent a small portion of the total p56^{lck} population. Also consistent with functional linkages between hsp90 and p56^{lck}, prolonged treatment of either LSTRA cells[3] or T cells (June et al., 1990) with herbimycin A leads to losses in the amount of p56^{lck} detectable on western blots. Together, these observations suggest that hsp90 chaperone machinery contributes to the folding of newly synthesized p56^{lck} in T cells and, by extension, may contribute to the *de novo* folding of other cellular members of the *src* family of nonreceptor tyrosine kinases as well.

Although our data indicate that hsp90 can have a second role in RRL, namely, prolonged maintenance of the structure of p56^{lck} via reiterative equilibrium interactions, we cannot at this time conclude that hsp90 provides similar prolonged reiterative maintenance for p56^{lck} in T cells. Physical interactions between hsp90 and p56^{lck} are not detected in membrane fractions of LSTRA cells (Figure 9), the site to which "mature" p56^{lck} molecules are believed to localize. CD4 capping experiments (Gassman et al., 1993) show co-localization of CD4 and activated p56^{lck}, but do not suggest co-localization of CD4 and hsp90.[4] The apparent contradictions between reiterative maintenance of p56^{lck} in RRL *versus* observations from T cells may reflect activities or events specific to T cells. Events occurring in T cells may stabilize p56^{lck} structure to an hsp90-independent state. Our utilization of the F505 mutation of p56^{lck} structure to facilitate kinase assays in RRL may also have contributed to a sustained requirement for reiterative hsp90 action. Although our current data suggest that p56^{lck} localized to the plasma membrane of T cells functions independently of hsp90 support, we cannot at this time rule out the possibility that p56^{lck} action at other subcellular localizations might require hsp90.

A requirement for stabilization events is consistent with one model for interactions between hsp90 machinery and steroid hormone receptors. This model postulates that steroid hormone binding competence is maintained via dynamic reiterative interactions with hsp90 machinery until hormone binding stabilizes the structure of the receptor such that it no longer interacts with hsp90 (Smith, 1993). Extrapolating from this model, reiterative support of kinase structure prior to stabilization events may represent a critical interface between p56^{lck} biogenesis and the regulation or localization of p56^{lck} kinase activity. We are currently examining potential roles for hsp90 in mediating p56^{lck} maturation and function in human T cells and the effects of geldanamycin on these putative roles and interactions.

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[3] S. D. Hartson, and R. L. Matts, unpublished observations.

[4] G. Zenner, and P. Burn, unpublished observations.

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