Association of Hsp90 with Cellular *Src*-Family Kinases in a Cell-Free System Correlates with Altered Kinase Structure and Function†

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ABSTRACT: Following synthesis in the cytoplasm, the transforming proteins encoded by the retroviral oncogenes *src*, *yes*, *fps*, *fes*, and *fgr* form complexes with hsp90 and the hsp90 cohort p50. These cytoplasmic complexes are intermediates in the production of the mature membrane-associated kinase. However, soluble complexes between the nascent cellular homologs of these proteins and hsp90-p50 have not been readily detected [Brugge, J. S. (1986) *Curr. Top. Microbiol. Immunol. 123*, 1–22 and references therein]. In this paper, we have utilized protein synthesis in reticulocyte lysate to determine whether three cellular members of the *src* family of tyrosine kinases, myeloid-specific p59^{fgr}, B cell-specific p59^{fgr}, and p56^{lck}, form complexes with hsp90. Following their synthesis, fast- and slow- sedimenting forms of these proteins can be separated on glycerol gradients. Anti-hsp90 monoclonal antibodies co-immunoadsorb the fast-sedimenting, but not the slow-sedimenting, forms of these kinases from gradient fractions. These hsp90 complexes can be detected in the complete absence of detergent. Conversely, an unrelated protein, firefly luciferase, does not form stable complexes with hsp90 following synthesis in reticulocyte lysate. Anti-p56^{lck} antibodies specifically co-immunoadsorb hsp90 from protein synthesis reactions programmed with *lck* RNA. The fast-sedimenting, complex-bound form of p56^{lck} is deficient in autophosphorylation activity and phosphorylates an exogenous substrate, acid-treated enolase, less efficiently than does the monomeric form. Fast-sedimenting p56^{lck} is hypersentitive to limited proteolysis by chymotrypsin. These results demonstrate that cellular members of the *src* family of tyrosine kinases, like the oncogenic viral members, form specific and stable complexes with hsp90 and provide an initial characterization of the structural and functional differences between hsp90-associated *versus* monomeric kinases.

In cells infected with Rous sarcoma virus, two cellular proteins have been suggested to function in the transport of p60$^{v\text{-}src}$ [1] to the plasma membrane (Courtneidge & Bishop, 1982). These proteins, hsp90[2] (Oppermann et al., 1981) and the hsp90 cohort p50 (Perdew & Whitelaw, 1991; Whitelaw et al., 1991; Georgopoulos, 1992), form complexes with the viral homologs of the *src*, *fps*, *yes*, *fes* [Brugge (1986) and references therein], and *fgr* gene products (Ziemiecki, 1986; Ziemiecki et al., 1986). The physiological significance of these complexes was established by pulse-labeling studies, which demonstrated that hsp90 and p50 are bound to nascent p60$^{v\text{-}src}$ in the cytoplasmic fractions of infected cells (Courtneidge & Bishop, 1982; Brugge et al., 1983). When complexed with hsp90 and p50, p60$^{v\text{-}src}$ is hypophosphorylated and deficient in kinase activity. After dissociation from hsp90 and p50, p60$^{v\text{-}src}$ is phosphorylated on tyrosine and serine, has kinase activity, and is associated with membrane fractions of infected cells. From these observations, it was suggested that hsp90 and p50 might function in the transport, myristylation, phosphorylation, and/or regulation of p60$^{v\text{-}src}$ [reviewed in Brugge (1986)]. Hsp90 plays an essential, positive role in the *in vivo* expression of p60$^{v\text{-}src}$ activity, as evidenced by compromised kinase function and activity in hsp90-deficient yeast (Xu & Lindquist, 1993).

The existence of complexes between hsp90 and viral homologs of the *src* family implies the existence of similar complexes between hsp90 and cellular *src*-family kinases. Consistent with this possibility, cellular proteins such as the heme-regulated inhibitor of protein synthesis (HRI), steroid hormone receptors (SHR), and the *raf* kinase gene product occur in complexes with hsp90 (Tai et al., 1986; Estes et al., 1987; Kost et al., 1989; Renoir et al., 1990; Sanchez et al., 1990a,b; Smith et al., 1990; Matts et al., 1992; Stancato et al., 1993). This conclusion is also supported by the ubiquity of hsp90- complexes among viral *src*-family kinases, since the ability to form such complexes is unlikely to result from convergent evolution of viral genes (Brugge, 1986). However, complexes between hsp90-p50 and p60$^{c\text{-}src}$ are difficult or impossible to detect in detergent lysates of untransformed cells that overexpress p60$^{c\text{-}src}$ [Schuh and Brugge, unpublished results, cited in Brugge (1986); Iba et al. (1985)]. Similarly, using immunological techniques, we have been unable to detect associations between hsp90 and the *src*-family kinase p56^{lck} in detergent-free lysates of resting murine T cells.[3]

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[1] Abbreviations: p60$^{v\text{-}src}$, the viral homolog of p60^{src}; p60$^{c\text{-}src}$, the cellular homolog of p60^{src}; hsp, heat shock protein; SHR, steroid hormone receptor; HRI, heme-regulated inhibitor (eIF2α kinase) of protein synthesis; mAb, monoclonal antibody; anti-lckCT, antibodies directed against the C-terminus of human p56^{lck}; anti-lckNT, antibodies directed against the N-terminus of human p56^{lck}; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBR, tumor-bearing rabbits; RSV, Rous sarcoma virus.

[2] These numbers are used for purposes of nomenclature and do not necessarily reflect the precise or relative molecular weights of these proteins in our hands.

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In vitro studies have demonstrated that mature $p60^{c\text{-}src}$ stripped from cell membranes with detergent will bind hsp90 and p50 upon subsequent incubation in reticulocyte lysate (Hutchison et al., 1992). However, the significance of reconstituted $p60^{c\text{-}src}$–hsp90 complexes is unclear, since interactions between hsp90 and $p60^{v\text{-}src}$ are specific for the nascent kinase *in vivo* (Brugge, 1986). It has been suggested that the reassociation of mature $p60^{c\text{-}src}$ with hsp90-p50 might result from the partial denaturation of the kinase by detergent, thus generating a folding intermediate subsequently recognized by hsp (Hutchison et al., 1992). Because these reconstituted complexes might thus represent generic associations between hsp and partially denatured protein, this approach yields somewhat ambiguous results. The inability to easily isolate authentic complexes between nascent cellular *src*-family kinases and hsp90-p50 has complicated efforts to determine what role, if any, hsp90 plays in the the function of these kinases.

To overcome the limitations inherent to the approaches discussed above, we have synthesized nascent *src*-family kinases in rabbit reticulocyte lysate to reexamine possible roles for hsp90 in modulating the structure and function of these kinases. We report here that three cellular members of the *src* family of tyrosine kinases, myeloid-specific $p59^{fgr}$ [2], B cell-specific $p59^{fgr}$, and $p56^{lck}$, associate with hsp90 following their synthesis in reticulocyte lysate. Conversely, an unrelated enzyme, firefly luciferase, does not form stable complexes with hsp90 following synthesis. Kinase assays and limited proteolysis demonstrate that nascent, hsp90-bound $p56^{lck}$ produced in reticulocyte lysate is functionally and structurally distinct from the monomeric form similarly produced.

EXPERIMENTAL PROCEDURES

Materials. The plasmids pfu2 (Patel & Brickell, 1993) and pfc11 (Brickell & Patel, 1988) containing the full-length coding sequence of the myeloid- and B cell-specific *fgr* gene products, respectively, were supplied by Dr. Paul Brickell (University College London Medical School). The plasmid pNT18, containing 2.1 kb of murine *lck* cDNA including the full-length *lck* coding sequence (Marth et al., 1985), was supplied by Dr. Roger Perlmutter (Howard Hughes Medical Institute, University of Washington). The plasmid pSP64T, containing untranslated 5′ and 3′ β-globin cDNA sequences interrupted by a single *Bgl*II linker and downstream from the SP6 RNA polymerase promoter (Kreig & Melton, 1984), was supplied by Dr. Douglas Melton (Harvard University). The plasmid pGEMluc, containing the full-length luciferase coding region cloned into pGEM3Z, was purchased from Promega. Plasmids pBluescript(KS+) and pBluescript(SK+) were purchased from Stratagene. Nuclease-treated rabbit reticulocyte lysate was purchased from Ambion (Retic Lysate IVT Translation Kit) or from Promega (TNT Coupled Reticulocyte Lysate System). Polyclonal anti-hsp90/hsp70 antiserum [4322 antiserum (Kreig & Melton, 1984)] was supplied by Dr. Stephen J. Ullrich (National Cancer Institute). Monoclonal antibody (mAb) directed against hsp90 [8D3 IgM mAb (Perdew & Whitelaw, 1991)] was provided by Dr. Gary H. Perdew (Purdue University). Polyclonal antibodies directed against the C-terminus (anti-lckCT) of human $p56^{lck}$ (residues 476–509) (Koga et al., 1986; Trevillyan et al., 1986) or against the N-terminus (anti-lckNT) of human $p56^{lck}$ (residues 22–51) were purchased from Upstate Biotechnology, Inc. Nonimmune control antibodies were purchased from Sigma.

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

Construction of Transcription Templates. The plasmid pfu2 contains 2.4 kb of myeloid-specific $p59^{fgr}$ cDNA, consisting of the full-length reading frame and untranslated flanking sequences, cloned into the *Eco*RI site of pBluescript-(SK+). pfu2 was digested with *Eco*RI and end-filled with Klenow enzyme in the presence of 0.1 mM each dCTP, dGTP, dTTP, and ATP, and the 2.4-kb restriction fragment was ligated to *Bgl*II-linearized pSP64T which had been similarly end-filled. The resulting plasmid, pSPM-fgr, contains myeloid-specific *fgr* cDNA nested between the pSP64T-derived 5′ and 3′ untranslated globin cDNA sequences in an orientation such that transcription with SP6 RNA polymerase yields a sense RNA transcript. Purified pSPM-fgr DNA was linearized with *Eco*RI prior to *in vitro* transcription. To produce transcription template for B cell-specific $p59^{fgr}$, the 2.0-kb *Eco*RI fragment of pfc11 containing the full-length B cell *fgr* open-reading frame and untranslated flanking DNA was subcloned into pSP64T as described for pSPM-fgr. Purified plasmid DNA was linearized with *Eco*RI prior to transcription.

To produce transcription template for firefly luciferase, the 1.7-kb *Bam*HI/*Xho*I fragment of pGEMluc was subcloned into the corresponding sites of pBluescript(KS+). This subcloning was necessary to remove an upstream AUG codon present in the multiple cloning site of pGEMluc. The resulting plasmid, pBSluc, contains the full-length luciferase open-reading frame in an orientation such that transcription with T7 RNA polymerase yields a sense RNA. Purified pBSluc DNA was linearized with *Xho*I prior to *in vitro* transcription. Alternatively, the 1.7-kb *Bam*HI/*Xho*I fragment of pGEMluc was subcloned into pSP64T as described for pSPM-fgr, and plasmid DNA was linearized with *Pst*1 before transcription.

To produce transcription template for $p56^{lck}$, the 1.6-kb *Stu*I fragment of pNT18 was ligated to pBluescript(SK+) DNA that had been linearized with *Kpn*I and digested with T4 DNA polymerase in the presence of 0.1 mM each dCTP, dGTP, dTTP, and ATP. The resulting plasmid, pBSlck, contains the full-length open-reading frame coding for murine $p56^{lck}$ in an orientation such that transcription with T7 RNA polymerase yields a sense RNA. Purified pBSlck DNA was linearized with *Xho*I prior to *in vitro* transcription. For high-efficiency expression of $p56^{lck}$, the *Stu*I fragment of NT18 was ligated to pSP64T DNA that had been linearized and end-filled as described for the construction of pSPM-fgr. The resulting plasmid, pSPlck, contains the full-length *lck* open-reading frame nested between globin untranslated sequences in an orientation such that transcription with SP6 RNA polymerase yields a sense RNA. Purified pSPlck DNA was linearized with *Eco*RI prior to *in vitro* transcription.

In Vitro Transcription and Translation. Capped RNA transcripts were produced in reactions containing 500 units/mL of the appropriate RNA polymerase (Promega), transcription buffer supplied with each polymerase, 50 μg/mL linearized DNA template, 0.3 mM $m^7G(5')ppp(5')G$ cap analog (Pharmacia), 0.12 mM GTP, 0.4 mM each CTP, ATP, and UTP, 10 mM DTT, and 200 units/mL RNasin (Promega). Following transcription at 37 °C for 2 h, reactions were digested with DNase, extracted with phenol/chloroform (5:1 v/v), and precipitated with 2.5 volumes of ethanol in the presence of 2 M ammonium acetate. RNA pellets were dissolved in water, reprecipitated with ethanol/ammonium acetate, and redissolved in water. Transcript size, integrity, and concentration were analyzed by electrophoresis on agarose gels. RNA was translated at 10–20 μg/mL for 20 min in nuclease-treated reticulocyte lysate according the the manufacturer's suggestions for capped RNAs. As controls, protein

synthesis reactions were also programmed with no RNA (water) or with luciferase RNA. Alternatively, p56^{lck} was synthesized by coupled transcription/translation using the Promega TNT lysate system. Translations were terminated after 20 min to ensure that rates of translation remained linear during the course of protein synthesis and to ensure that translation reactions contained a large proportion of newly synthesized protein species. Protein synthesis was monitored by measuring the incorporation of L-[^{35}S]Met (NEN/DuPont) into TCA-insoluble material, and aliquots of translation reactions were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described (Matts et al., 1992).

Gradient Analysis and Immunoadsorption of Protein Complexes. Translation reactions were diluted 1:2 (v/v) with 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 0.2 mM EDTA, and 2 mM magnesium acetate, layered over linear 10–30% (v/v) glycerol gradients containing this same buffer, and centrifuged for 18.5 h at 45 000 rpm in a Sorvall AH-650 rotor, or alternatively, for 24.4 h at 40 000 rpm in a Beckman SW 50.1 rotor. Sedimentation size standards (Pharmacia) were analyzed on a parallel gradient. Gradients were fractionated (0.2 mL per fraction) from the bottom, and aliquots of odd-numbered fractions were analyzed on 10% polyacrylamide gels. Gels were transfered to PVDF membranes (Bio-Rad) as previously described (Matts et al., 1992), and membranes were exposed to X-ray film. The intensities of bands corresponding to authentic protein species were analyzed by densitometry of appropriately exposed autoradiograms using a PDI Digital Imaging System.

Gradient fractions containing slow- or fast-sedimenting radiolabeled protein peaks were diluted 1:1 (v/v) with 20 mM Tris·HCl, and hsp90 or hsp90 complexes were immunoadsorbed under detergent-free conditions as previously described (Matts et al., 1992) using the 8D3 monoclonal anti-hsp90 IgM. Control immunoadsorptions with nonimmune monoclonal IgM were also performed. Prior to the immunoadsorption of p56^{lck} directly from protein synthesis reactions, 25 μL of synthesis reaction was adjusted to 1% (v/v) Triton X-100 and 0.25% (w/v) sodium deoxycholate using a 4× detergent stock to reduce nonspecific binding of nascent p56^{lck} to resins and tubes. These diluted lysates were incubated on ice for 3 h with 100 μg of polyclonal anti-lckCT, anti-lckNT, or nonimmune antibodies, and immune complexes were isolated by binding to 20 μL of protein G–Sepharose (Pharmacia) for 1 h on ice. Immunoadsorbed pellets were washed twice with 20 mM Tris·HCl (pH 7.4) and 150 mM NaCl containing 1× detergent, once with 20 mM Tris·HCl (pH 7.4) and 500 mM NaCl containing 1× detergent, and three more times with 20 mM Tris·HCl (pH 7.4) and 150 mM NaCl containing 1× detergent. Immunoadsorbed pellets were then analyzed on 10% polyacrylamide gels. Following transfer of gels to PVDF membranes, hsp90 and hsp70 were detected by western blotting as described (Matts et al., 1992) using polyclonal anti-hsp90/hsp70 antisera.

Kinase Assays. For kinase assays, p56^{lck} was hyperexpressed in translation reactions, and reactions were sedimented on glycerol gradients. Anti-lckCT antibodies were used as described above to immunoadsorb p56^{lck} from pooled gradient fractions corresponding to fast- or slow-sedimenting peaks of radiolabeled protein. Immunoadsorption pellets containing anti-lckCT immune complexes were washed three additional times with 20 mM Tris·HCl (pH 7.4) and 150 mM NaCl, and aliquots of the pellets (1/20 of the total pellet) were analyzed by SDS–PAGE to assess the efficiency of immunoadsorption. To detect autophosphorylation of p56^{lck}, separate aliquots of the pellets (1/20 of the total pellet) were incubated in 15 μL of 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 10 mM $MnCl_2$, 10 μM [γ-^{32}P]ATP (specific activity 100 Ci/mmol) (NEN/DuPont) for 10 min at 30 °C. Reactions were terminated by the addition of SDS sample buffer and subjected to SDS–PAGE. Following transfer of gels to PVDF membranes, membranes were treated with 1 N KOH for 45 min at 55 °C, and phosphorylated proteins were detected by autoradiography at −70 °C with an intensifying screen. To assay kinase activity of p56^{lck} using an exogenous substrate, aliquots of immunoadsorbed pellets (18/20 of the total pellet) were incubated in 30 μL of 0.5 μg/μL acid-denatured enolase (Cooper et al., 1984), 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 10 mM $MnCl_2$, and 1.0 mM [γ-^{32}P]ATP (specific activity 1.0 Ci/mmol) (NEN/DuPont). After 10 min at 30 °C, the reaction was briefly centrifuged and 10 μL of the supernatant analyzed by SDS–polyacrylamide electrophoresis, transfer to PVDF membranes, and base hydrolysis as described above for autophosphorylation assays. Phosphorylated enolase was detected by autoradiography at −70 °C with an intensifying screen.

Proteolysis Assays. To generate substrates for proteolysis assays in which glycerol and hemoglobin contents were equivalent, heavy or light gradient fractions corresponding to fast- or slow-sedimenting peaks of radiolabeled protein were prepared as described above to isolate hsp90-bound or free monomeric p56^{lck}, respectively. Equivalent gradient fractions from translation reactions programmed with no RNA (control fractions) were similarly prepared. Equal volumes of heavy gradient fraction lacking (control) or containing hsp90-bound p56^{lck} were mixed with light gradient fractions containing or lacking (control) monomeric p56^{lck}, respectively. Such mixing resulted in "control-pooled" gradient fractions differing only in the species of p56^{lck} present. For proteolysis, 1 volume of control-pooled fraction was mixed with 1 volume of 20 mM Tris·HCl, 150 mM NaCl, 4.0 mM $CaCl_2$, 0.1 mM EDTA, and 200 μg/mL chymotrypsin and incubated on ice for the indicated times. Digestions were terminated by the addition of soybean trypsin/chymotrypsin inhibitor, incubated on ice an additional hour, and analyzed by SDS–PAGE on 10% gels.

RESULTS

Cellular p59^{fgr} Forms Complexes with Hsp90. Because the protein product of the viral *fgr* gene has been demonstrated to form very stable complexes with hsp90 (Ziemiecki, 1986; Ziemiecki et al., 1986), we attempted to demonstrate that the cellular *fgr* gene product formed similar complexes with hsp90 following synthesis in a cell-free system. Myeloid-specific *fgr* RNA was synthesized *in vitro* and used to program protein synthesis reactions. Assuming endogenous methionine content of lysate to be approximately 3.5 μM, protein synthesis reactions produced 0.06 μg of [^{35}S]-protein per mL of reaction (not shown). Following SDS–PAGE, the major protein product was identified as the *fgr* gene product (p59^{fgr})[2] on the basis of its RNA specificity and apparent MW. A minor protein product with a M_r of 6.6×10^4 was also produced (not shown). To determine if p59^{fgr} occurred in high-weight protein complexes, translation reactions were separated by sedimentation through glycerol gradients and aliquots of gradient fractions were analyzed by SDS–PAGE (Figure 1A). Densitometry of appropriately exposed autoradiograms (Figure 1B) indicated that approximately 45% of the p59^{fgr} synthesized in the cell-free system occurred as a monomer cosedimenting

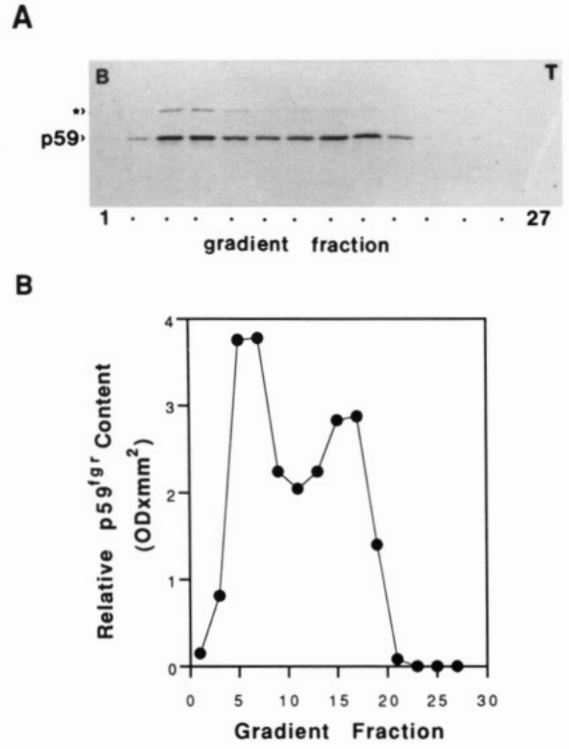

FIGURE 1: Sedimentation of monomeric and complex-bound *fgr* translation products. Following *in vitro* synthesis of [^{35}S]p59^{fgr} and sedimentation of the translation reaction through a glycerol gradient, aliquots of odd-numbered fractions were analyzed by SDS–PAGE and autoradiography (panel A). Gradients were fractionated from the bottom (B) to the top (T) of the gradient. The major (p59) and minor (★) *fgr* translation products are indicated. The relative amount of the major *fgr* translation product present in each gradient fraction was quantitated by densitometry (panel B).

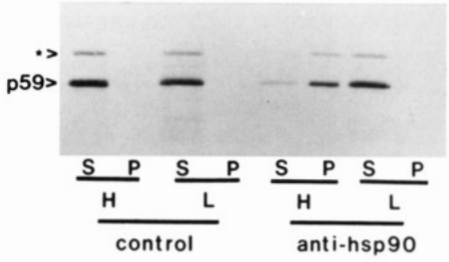

FIGURE 2: Specific immunoadsorption of fast-sedimenting *fgr* translation products with anti-hsp90 antibody. Nonimmune (control) or 8D3 (anti-hsp90) antibodies were used in immunoadsorption reactions with heavy gradient fractions (H) containing fast-sedimenting *fgr* translation products or with light gradient fractions (L) containing slow-sedimenting *fgr* translation products. The nonadsorbed supernatants (S) and the adsorbed pellets (P) were subsequently analyzed by SDS–PAGE and autoradiography. The major (p59) and minor (★) *fgr* translation products are indicated.

with hemoglobin, while the balance of the p59^{fgr} population sedimented as a discrete peak with an approximate M_r of 2.0 $\times 10^5$. Western blotting of gradient fractions with anti-hsp90/hsp70 antiserum demonstrated that hsp90 and hsp70[2] were present in gradient fractions corresponding to both p59^{fgr} peaks (not shown). Three observations suggested that the fast-sedimenting peak did not represent artifactual nonspecific aggregation: (1) fast-sedimenting p59^{fgr} occurred in a discrete peak well-resolved from the bottom of the gradient; (2) less than 1% of the full-length protein was recovered as pelleted material; (3) this peak occurred at a position in the gradient corresponding to that previously reported (Brugge, 1986) for complexes between hsp90-p50 and p60$^{v\text{-}src}$.

To determine if the fast-sedimenting form of p59^{fgr} was complexed with hsp90, fast-sedimenting gradient fractions were immunoadsorbed with 8D3 anti-hsp90 mAb. Three controls were utilized to ensure that recovery of p59^{fgr} with the anti-hsp90 mAb was specific for the presence of hsp90: (1) hsp90 complexes were eluted from the low-affinity 8D3 anti-hsp90 mAb using high salt (Perdew & Whitelaw, 1991) rather than SDS to prevent recovery of proteins which bound to the antibody or resin in a nonspecific fashion; (2) immunoadsorptions with nonimmune antibodies served as additional controls for nonspecific binding; and (3) slow-sedimenting gradient fractions were also analyzed to ensure that binding of p59^{fgr} to anti-hsp90 mAb did not result from nonspecific binding or from cross-reactivity between the mAb and p59^{fgr}. Following immunoadsorption with nonimmune mAb, neither hsp90 (not shown) nor p59^{fgr} (Figure 2, control) present in heavy or light gradient fractions was recovered in the immunoadsorbed pellets, demonstrating that neither hsp90 nor fast- or slow-sedimenting p59^{fgr} was recovered from IgM–Sepharose pellets in a nonspecific fashion. Similarly, although anti-hsp90 mAb removed most of the slow-sedimenting hsp90 present in light gradient fractions (not shown), little or no slow-sedimenting p59^{fgr} was recovered in the pellet immunoadsorbed from light gradient fractions (Figure 2, anti-hsp90, L). Thus, neither cross-reactivity nor nonspecific binding between the anti-hsp90 mAb and p59^{fgr} was apparent. In contrast, when anti-hsp90 mAb was used to immunoadsorb complexes containing fast-sedimenting hsp90 from heavy gradient fractions, both p59^{fgr} (Figure 2, anti-hsp90, H) and hsp90 (not shown) were nearly quantitatively (>90%) removed from the immunoadsorption supernatant. Thus, the discrete fast-sedimenting myeloid-specific p59^{fgr} peak represented a homogeneous population of kinase molecules occurring in complexes with hsp90 following synthesis in reticulocyte lysate. Similarly, the protein product of B cell-specific *fgr* RNA formed complexes with hsp90 following synthesis in reticulocyte lysate (not shown). Since complexes between hsp90 and nascent p59^{fgr} were detected in the complete absence of detergent, these complexes were not detergent artifacts (Denis & Gustafsson, 1989).

Formation of Complexes Is Specific for p59^{fgr}. To rule out the possibility that stable associations between hsp90 and nascent proteins were a general phenomenon following protein synthesis in reticulocyte lysate, protein synthesis reactions were programmed with RNA coding for an unrelated protein, firefly luciferase, and analyzed on glycerol gradients. Luciferase was chosen as a control for two reasons: (1) we have observed that luciferase is quite sensitive to denaturation by heat shock, thus providing a convenient control for stress-induced denaturation and association with hsp's (Shumacher et al., 1994); and (2) with a molecular mass of 62 kDa, luciferase would sediment at a rate approximately equal to that of monomeric p59^{fgr}. Protein synthesis reactions programmed with luciferase RNA produced 0.2 μg/mL [^{35}S]-protein (not shown), consisting of single major protein species and several less-than-full-length species (not shown). The major species was identified as authentic luciferase on the basis of its RNA specificity and its M_r, while the less-than-full-length species probably arose due to the nonideal context of the firefly luciferase start codon (deWet et al., 1987). Unlike protein synthesis reactions programmed with *fgr* RNA, reactions programmed with luciferase RNA did not produce detectable minor 66-kDa species. When protein synthesis reactions were analyzed on glycerol gradients, luciferase sedimented only as a monomer (Figure 3). Similar sedimentation profiles were obtained when luciferase RNA containing 5′ and 3′ untranslated globin cDNA sequences

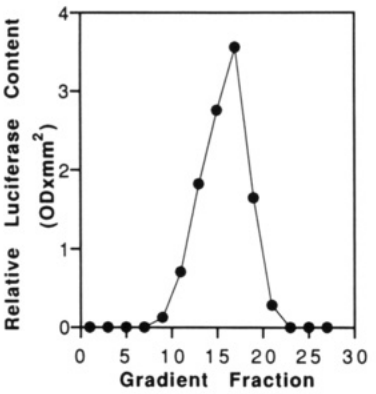

FIGURE 3: Sedimentation of monomeric luciferase. Following *in vitro* synthesis of [^{35}S]luciferase and sedimentation of the translation reaction through a glycerol gradient, gradients were fractionated from the bottom (fraction 1) and aliquots of odd fractions were analyzed by SDS–PAGE and autoradiography. The relative amount of the major luciferase translation product present in each gradient fraction was quantitated by densitometry of an appropriately exposed autoradiogram.

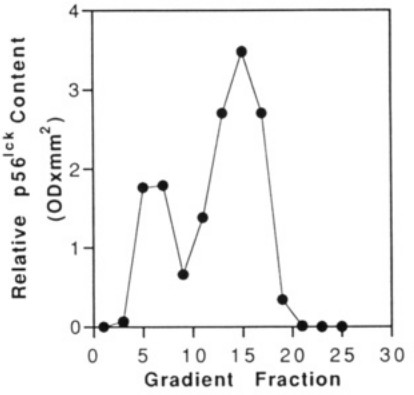

FIGURE 4: Sedimentation of monomeric and complex-bound *lck* translation products. Following *in vitro* synthesis of [^{35}S]p56^{lck} and sedimentation of the translation reaction through a glycerol gradient, gradients were fractionated from the bottom (fraction 1), and aliquots of odd fractions were analyzed by SDS–PAGE and autoradiography. The relative amount of the single major *lck* translation product present in each gradient fraction was quantitated by densitometry of an appropriately exposed autoradiogram.

was translated (not shown). Thus, luciferase did not form stable complexes with hsp90, indicating that the observed complexes between hsp90 and p59^{fgr} represented a property of p59^{fgr} rather than a general property of protein synthesis in reticulocyte lysate. This observation was consistent with the suggestion by Xu and Lindquist (1993) that hsp90 function is highly selective and specific *in vivo*.

p56^{lck} Forms Complexes with Hsp90. To determine if the formation of complexes with hsp90 might be ubiquitous among cellular *src*-family kinases, we extended our analysis to another member of the *src* family, p56^{lck}. We selected p56^{lck} for more extensive analysis of hsp90 complexes for five reasons: (1) no viral homolog for p56^{lck} has been reported; (2) *lck* cDNA contains unique restriction sites flanking the coding sequence, thus facilitating the construction of subclones for hyperexpression of the *lck* gene product (Marth et al., 1985); (3) monospecific antisera raised against specific portions of the p56^{lck} sequence were readily available; (4) a great deal is known about structure–function relationships for p56^{lck}, enabling us to interpret our results in the context of these relationships; and (5) infection of T cells by human immunodeficiency virus may affect p56^{lck} function (Juszczak et al., 1991; Cefai et al., 1992; Crise & Rose, 1992). When RNA was transcribed from the pBSlck template and used to program protein synthesis reactions, 0.1 μg of [^{35}S]-protein was produced per milliliter of reaction (not shown). The major [^{35}S]-protein produced was identified as the *lck* gene product on the basis of its RNA specificity and apparent M_r (not shown). As was observed for *fgr* RNA, reactions programmed with *lck* RNA also synthesized a minor RNA-specific protein species with an M_r of 6.6×10^4 (not shown). Because *in vitro*-transcribed *lck* RNA contained only the *lck* coding region and 51 nucleotides of flanking sequence, this minor species arose from posttranslational modifications rather than from utilization of alternative start or stop codons. A minor species with a similar M_r is observed when authentic *lck* mRNA isolated from LSTRA cells is translated in rabbit reticuloctye lysate; like our minor species, the origin of these minor *lck* species remains unknown (Marth et al., 1985). Following gradient analysis, fast- and slow-sedimenting species of p56^{lck} were apparent (Figure 4), only the heavier of which was immunoadsorbed with anti-hsp90 (Figure 5). Additionally, p56^{lck} could be immunoadsorbed in an immune-specific fashion directly from protein synthesis reactions with anti-hsp90 (not shown). Thus, p56^{lck}, like p59^{fgr}, forms complexes with hsp90 following synthesis in reticulocyte lysate.

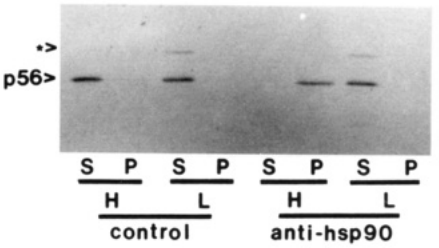

FIGURE 5: Specific immunoadsorption of fast-sedimenting *lck* translation products with anti-hsp90 antibody. Nonimmune (control) or 8D3 (anti-hsp90) antibodies were used in immunoadsorption reactions with heavy gradient fractions (H) containing fast-sedimenting *lck* translation products or with light gradient fractions (L) containing slow-sedimenting *lck* translation products. The nonadsorbed supernatants (S) and the adsorbed pellets (P) were subsequently analyzed by SDS–PAGE and autoradiography. The major (p56) and minor (★) *lck* translation products are indicated.

To hyperexpress the *lck* gene product in reticulocyte lysate, *lck* RNA containing flanking untranslated globin cDNA sequences was transcribed from the plasmid pSPlck. These untranslated globin sequences enhanced expression of p56^{lck} 20-fold. When analyzed by SDS–PAGE, translation reactions hyperexpressing p56^{lck} produced a major RNA-specific protein doublet (not shown). Additionally, the minor protein species of 66 kDa was again produced. The p56^{lck} doublet produced in this cell-free system probably represents kinase molecules varying in the phosphorylation states of individual Ser residues (Casnellie & Lamberts, 1986; Veillette et al., 1988; Danielian et al., 1989). We have not further characterized the 66-kDa species due to distortion of its electrophoretic migration by abundant lysate proteins of similar sizes. For the major p56^{lck} doublet, sedimentation profiles and anti-hsp90 co-adsorption indicated that both electrophoretic forms were associated with hsp90 (not shown). The distribution of each electrophoretic species between hsp90-bound and monomeric states was equivalent (not shown). Similarly, hyperexpression of p56^{lck} did not appear to significantly affect the distribution of p56^{lck} between hsp90-bound and monomeric states (not shown). Although variations in the hsp90-bound *versus* monomer distribution were occasionally observed between different lots of reticulocyte lysate and among individual experiments, we have been unable to discern the molecular basis for these variations.

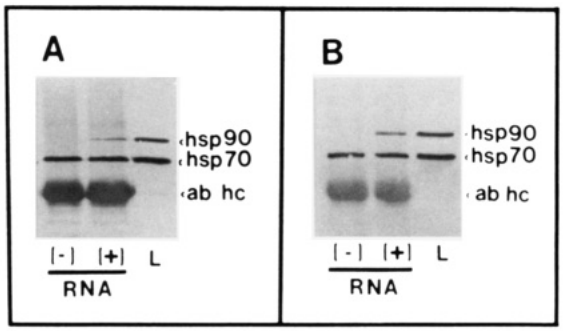

FIGURE 6: Co-adsorption of hsp90 from translation reactions by anti-p56^{lck} antibodies. Translation reactions programmed with water (–RNA) or with *lck* RNA (+RNA) were subjected to immunoadsorption with antibodies raised against peptides corresponding to the N-terminus (panel A) or the C-terminus (panel B) of human p56^{lck}. Following SDS–PAGE of immunoadsorbed pellets and electrotransfer to PVDF membranes, hsp90 (hsp90) and hsp70 (hsp70) were detected by western blotting with anti-hsp90/hsp70 antisera. Reticulocyte lysate (L) was also applied to the gel to supply a standard for the detection of hsp90 and hsp70. The heavy chain (ab hc) of the immunoadsorbing anti-lck antibodies detected by the secondary antibody is also indicated. Figure is a composite from two separate experiments.

Since reconstituted complexes between hsp90 and p60$^{v\text{-}src}$ have been reported to be stabilized by molybdate (Hutchison et al., 1992), complexes generated between hsp90 and nascent p56^{lck} following *in vitro* translation were analyzed following dilution and sedimentation in the presence of 20 mM sodium molybdate. Following such analysis, minimal differences were apparent in the relative distribution of the kinase population between heavy and light gradient fractions relative to those seen in the absence of molybdate, suggesting that molybdate was not necessary to stablize these complexes (not shown). However, when analyzed on glycerol gradients containing RIPA detergents, the whole kinase population was found to sediment as monomer, suggesting that the hsp90-p56^{lck} complex was unstable in the presence of detergent (not shown). The factor(s) which affect the relative distribution of p56^{lck} between the hsp90-bound and monomeric states are under further investigation.

As further proof that complexes were formed between hsp90 and p56^{lck} *in situ*, antibodies directed against p56^{lck} were used to co-immunoadsorb hsp90 directly from translation reactions. p56^{lck} was immunoadsorbed directly from translation reactions because western blots lacked the sensitivity necessary to readily detect hsp90 in anti-p56^{lck} immunoadsorptions from gradient fractions (not shown). Antibodies directed against residues 22–51 of human p56^{lck} (anti-lckNT) were selected because two observations suggested that the N-terminus of complex-bound p56^{lck} would be available for antibody binding: (1) deletion of the N-terminus of p60$^{v\text{-}src}$ does not abrogate the formation of complexes with hsp90-p50 (Cross et al., 1984); and (2) the various cellular and viral members of the *src* family have unique N-termini (Bolen et al., 1991), suggesting that a conserved hsp90-binding domain is not present in this region of the protein. Anti-lckNT antibodies immunoadsorbed p56^{lck} from translation reactions in an immunospecific fashion (not shown). When anti-lckNT immunoadsorbed pellets containing p56^{lck} were analyzed by western blotting with anti-hsp90/hsp70 antisera, two bands corresponding to hsp90 and hsp70 were detected (Figure 6A, +RNA). Hsp90 was not immunoadsorbed from protein synthesis reactions programmed with water (Figure 6A, –RNA), demonstrating that immunoadsorption of hsp90 was specific for the presence of p56^{lck} and did not result from nonspecific binding of hsp90 to the antibody or resin. In contrast to this specific adsorption of hsp90, binding of hsp70 was not RNA-specific (Figure 6A, –RNA) and probably represented nonspecific binding of hsp70 to antibody, resin, or both (Scherrer et al., 1990; Hutchison et al., 1992). Due to this nonspecific binding, we were unable to demonstrate the presence of hsp70 in the complex between p56^{lck} and hsp90, as has been reported for p60^{src} (Hutchison et al., 1992). Nonetheless, p56^{lck}-specific co-adsorption of hsp90 with anti-lckNT antibodies confirmed that p56^{lck} formed complexes with hsp90 and demonstrated that at least some epitopes within the unique N-terminus of p56^{lck} were available for antibody binding.

Unlike the N-terminal domain, the C-terminal domain of *src*-family kinases has been implicated in the formation of complexes with hsp90 since antibodies directed against the six C-terminal residues of p60$^{v\text{-}src}$ (residues 521–526) co-immunoadsorb hsp90 and p50 very poorly or not at all (Sefton & Walter, 1982). Thus, to determine whether epitopes within the C-terminal domain of p56^{lck} might be blocked by the binding of hsp90, antibodies directed against the 34 C-terminal residues (residues 476–509) of human p56^{lck} (anti-lckCT antibodies) were used to co-immunoadsorb hsp90 from translation reactions. As was observed for antibodies directed against the N-terminus, p56^{lck}-specific co-adsorption of hsp90 with anti-lckCT antisera (Figure 6B) confirmed that p56^{lck} formed complexes with hsp90. Thus, in contrast to p60$^{v\text{-}src}$ (Sefton & Walter, 1982), at least some epitopes within the conserved C-terminus of hsp90-bound p56^{lck} were available for antibody binding.

Complex-Bound p56^{lck} Is Deficient in Kinase Activity. Because the physiological relevance of complexes between hsp90 and p60$^{v\text{-}src}$ has been established, in part, by the demonstration that complex-bound p60$^{v\text{-}src}$ is deficient in kinase activity (Brugge, 1986), we wished to similarly demonstrate such a difference for complex-bound *versus* monomeric p56^{lck}. p56^{lck} was hyperexpressed in protein synthesis reactions, and the fast- and slow-sedimenting forms were separated on glycerol gradients. When heavy and light gradient fractions were immunoadsorbed with anti-lckCT antibodies, equal amounts of p56^{lck} were recovered in the immunoadsorbed pellets (Figure 7A), although the efficiencies of immunoadsorption from heavy *versus* light gradient fractions were not equal (20% *versus* 40% recovered, respectively). This unequal efficiency may reflect steric hindrance by hsp90 of antibody binding or might simply reflect the unequal glycerol and protein compositions of heavy *versus* light gradient fractions. Incubation of the immunoadsorbed p56^{lck} in the presence of [γ-^{32}P]-ATP resulted in the appearance of a [^{32}P]-protein species which comigrated with the slower electrophoretic form of p56^{lck} (Figure 7B, lck RNA). However, this species was not apparent when control immunoadsorbed pellets were assayed (Figure 7B, no RNA). Thus, appearance of this band was interpreted to represent autophosphorylation of p56^{lck}. For the data shown, densitometry suggested that the autophosphorylation activity observed for monomeric p56^{lck} was 3-fold greater than that observed for complex-bound p56^{lck} (Figure 7B, L *versus* H, respectively). In addition to autophosphorylated p56^{lck}, a protein species with a M_r of 50 000 was sometimes phosphorylated during kinase assays of complex-bound p56^{lck}; however, phosphorylation of this protein was not always reproducible among separate experiments. Perhaps this band represented phosphorylation of the hsp90 cohort p50, as has been reported for p60$^{v\text{-}src}$ *in vivo* (Brugge, 1986).

Because autophosphorylation assays did not adequately address the possibility that phosphorylation of p56^{lck} was due to contamination of immune complexes by a kinase endogenous

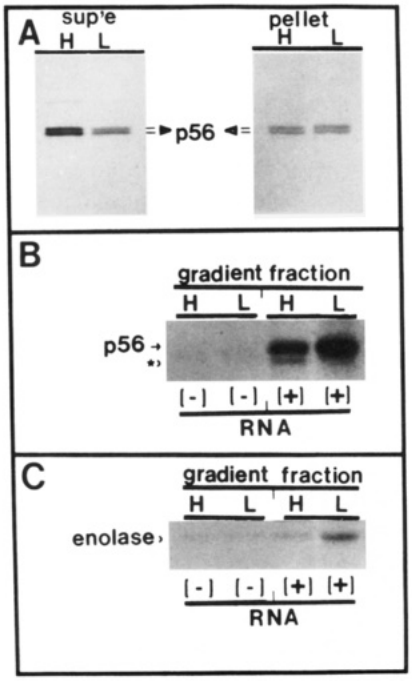

FIGURE 7: Kinase assays of fast- or slow-sedimenting p56^{lck} produced *in vitro*. Translation reactions containing [^{35}S]p56^{lck} (+ RNA) were separated on glycerol gradients and heavy gradient fractions (H) containing fast-sedimenting *lck* translation products or light gradient fractions (L) containing slow-sedimenting *lck* translation products were pooled. Translation reactions programmed with water were similarly separated on gradients to provide equivalent pooled control gradient fractions (–RNA). Gradient fractions were then subjected to immunoadsorptions with anti-lckCT antibodies. After adsorption and washing, each immunoadsorption pellet was resuspended, divided into three aliquots for analysis as described for individual panels, and repelleted. To ensure that equal amounts of hsp90-bound and monomeric p56^{lck} were compared, one set of aliquots of the immunoadsorption reactions was analyzed by SDS–PAGE and autoradiography of [^{35}S]species (panel A). *Lck* translation products (p56) present in the nonadsorbed supernatants (sup'e) or in the adsorbed pellets (pellet) are indicated. Autophosphorylation of immunoadsorbed *lck* translation product in the presence of [γ-^{32}P]-ATP was analyzed by SDS–PAGE and autoradiography (panel B). [^{32}P]*lck* translation product (p56) and 50-kDa protein (★) are indicated. Phosphorylation of enolase was detected following incubation of immunoadsorbed pellets in the presence of [γ-^{32}P]ATP and acid-treated enolase (panel C). Figure is a composite from separate membranes exposed as a single autoradiogram.

to reticulocyte lysate, we assayed kinase activity in anti-lckCT immunoadsorbed pellets using an exogenous substrate, acid-treated enolase (Figure 7C). For the data shown, phosphorylation of enolase was enhanced 3-fold over the no-RNA background by monomeric p56^{lck} while p56^{lck} isolated from heavy gradient fractions enhanced the phosphorylation of enolase only approximately 1.6-fold, confirming that that p56^{lck} occurring in hsp90 complexes lacked full kinase activity. The activity of *src*-family kinases (Kypta et al., 1990), including p56^{lck} (Marie-Cardine et al., 1992; Nadeau et al., 1993), is enhanced to similar extents following activation of cells by various stimuli.

Complex-Bound and Monomeric p56^{lck} Have Different Conformations. Several hypotheses for hsp90 function propose that hsp90 stabilizes bound proteins in folding conformations which differ from those assumed in the absence of hsp90. To determine if the conformation of hsp90-bound p56^{lck} differed from that of the free monomer, both were subjected to limited proteolysis by chymotrypsin (Figure 8). Limited proteolysis reproducibly yielded well-defined discrete "fingerprints" for both monomeric and hsp90-bound p56^{lck}, consistent with the suggestion that the fast-sedimenting peak of [^{35}S]p56^{lck} was

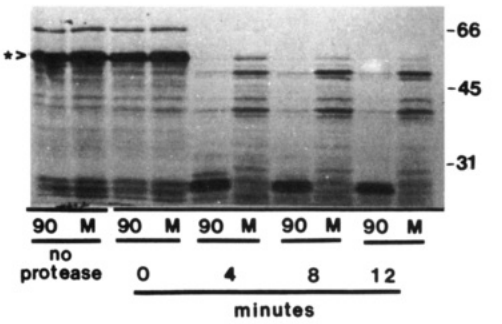

FIGURE 8: Limited chymotryptic digestion of hsp90-bound *versus* monomeric p56^{lck}. Hsp90-bound (90) and monomeric (M) p56^{lck} synthesized in reticulocyte lysate were separated on gradients and pooled with control gradient fractions as described in Experimental Procedures. Samples were digested with chymotrypsin for the indicated times (minutes), digestions were terminated by the addition of soybean trypsin–chymotrypsin inhibitor, and digestion products were separated by SDS–PAGE. The full-length translation product (★) is indicated. Molecular masses (kDa) and migrations of size standards are indicated along the right side of the figure. Figure is an autoradiogram.

comprised of a homogeneous population of kinase molecules. Hsp90-bound p56^{lck} was hypersensitive to chymotryptic digestion relative to the free monomer, demonstrating that the hsp90-bound kinase contained a region(s) of structure that, relative to the monomer, was more vulnerable to the proteolytic cleavage. Thus, the conformation assumed by hsp90-bound p56^{lck} differed from that of the monomeric kinase.

DISCUSSION

Evidence for Complexes between Hsp90 and Cellular Homologs of Src-Family Kinases. Historically, four approaches have been used to demonstrate the existence of complexes between p60$^{v\text{-}src}$ and hsp90-p50 [Brugge (1986) and references therein]: (1) antisera from rabbits bearing RSV-induced tumors (TBR antisera) and mAbs specific for p60$^{v\text{-}src}$ co-immunoadsorb hsp90 and p50 from lysates of RSV-infected cells; (2) p60$^{v\text{-}src}$ sediments as two forms, a slow-sedimenting monomer and a fast-sedimenting complex with an M_r of approximately 2.0 × 10^5; (3) hsp90 and p50 cosediment with fast-sedimenting p60$^{v\text{-}src}$ and can be co-immunoadsorbed from heavy gradient fractions with TBR antisera; and (4) polyclonal antisera and monoclonal antibodies directed against hsp90 co-immunoadsorb p60$^{v\text{-}src}$ and p50 from heavy gradient fractions and from lysate of RSV-infected cells. We have used two of these criteria to demonstrate complexes between hsp90 and the gene products of three cellular members of the *src* family, namely, that p56^{lck} and the myeloid- and B cell-specific forms of p59^{fgr} sediment as monomers and as 200-kDa complexes (Figures 4 and 1) and that anti-hsp90 antibodies co-immunoadsorb the fast-sedimenting forms of these enzymes (Figures 5 and 2). Also, we have fulfilled two additional criteria for the existence of complexes between p56^{lck} and hsp90, namely, that immunoadsorption of p56^{lck} from lysate with anti-p56^{lck} antibodies co-adsorbs hsp90 (Figure 6) and that immunoadsorption of hsp90 from lysate with anti-hsp90 mAb co-adsorbs p56^{lck} (not shown). On the basis of these criteria, we conclude that complexes containing hsp90, and by inference, p50, are formed with these cellular *src*-family kinases following their translation in reticulocyte lysate.

This conclusion implies the formation of similar complexes *in vivo*, consistent with the ubiquity of the *in vivo* interaction with hsp90 among viral and cellular kinases. However, complexes between hsp90-p50 and cellular p60^{src} are unde-

tectable (Iba et al., 1985) or are detected at very low levels [Schuh and Brugge, unpublished results, cited in Brugge (1986)] in detergent lysates of untransformed cells that overexpress $p60^{c\text{-}src}$. Thus, complexes formed between hsp90 and viral *src* homologs would appear to differ from those formed with the cellular homologs. This difference might result from a less stable interaction with hsp90 that would complicate detection of such complexes (Iba et al., 1985; Brugge, 1986). Consistent with this explanation, complexes between hsp90 and $p56^{lck}$ are unstable in the presence of detergents traditionally used in RIPA buffer (not shown), whereas complexes between hsp90 and $p60^{v\text{-}src}$ have been reported to be stable under these conditions (Brugge, 1986). This difference suggests that complexes between hsp90 and cellular *src*-family kinases may dissociate following detergent lysis of cells.

An alternative explanation for failures to detect complexes beween hsp90 and cellular $p60^{src}$ is that, *in vivo*, only a small portion of the cellular kinase population may occur in complexes with hsp90-p50 at a given time, perhaps reflecting an increased rate of turnover for the complex or an increased half-life for monomeric $p60^{c\text{-}src}$ (Brugge, 1986). In contrast to such a situation, a very high proportion of the [^{35}S]-protein population produced in reticulocyte lysate is nascent and this population is easily accessible, requiring no extended lysis or differential centrifugation prior to gradient analysis or immunoprecipitation. Additionally, heteromeric complexes containing hsp90 may be especially long-lived in reticulocyte lysate, perhaps due to the the absence or exhaustion of cellular factors which facilitate the maturation hsp90–kinase complexes and/or the activation of kinase activity.

Role of Hsp90–Kinase Complexes. Similarities between the hsp90 family and other stress inducible proteins suggest that hsp90 may act as a chaperone to stabilize protein folding intermediates (Gething & Sambrook, 1992), perhaps in conjunction with intracellular protein trafficking (Pratt, 1993). Consistent with this model, hsp90 is thought to enhance the binding of steroid hormones to SHR by holding SHR in a high-affinity binding conformation (Yamamoto et al., 1988; Bresnick et al., 1989). This conformation may represent a partially unfolded SHR protein molecule (Picard et al., 1988; Smith et al., 1990). However, for *src*-family kinases, the folding statuses of the hsp90-bound and monomeric proteins have not previously been compared. Our results demonstrate a distinct difference between the conformation of hsp90-bound $p56^{lck}$ and that of the monomer (Figure 8). The nature of this difference, namely, hypersensitivity to chymotrypsin, is consistent with a less-ordered, or partially unfolded, conformation for the kinase. Additionally, this structural difference correlates with a functional difference, namely, the observed deficiency in kinase activity of hsp90-bound $p56^{lck}$ (Figure 7). Thus, these observations support a model in which hsp90 chaperones the folding of kinase molecules.

For $p60^{v\text{-}src}$, the role of hps90 has been proposed to be obligate, processive, and productive (Brugge, 1986; Xu & Lindquist, 1993). However, such a role for hsp90 in $p56^{lck}$ maturation in reticulocyte lysate cannot be determined by the data presented here. Although complexes between hsp90 and $p56^{lck}$ may repesent an obligate intermediate in the folding pathway to the mature kinase, our data are equally consistent with a model in which hsp90 recognizes and binds to misfolded proteins resulting from nonproductive folding pathways or from protein denaturation. Thus, the observed binding of hsp90 to $p56^{lck}$ might represent either a cause or an effect of altered kinase conformation. Nonetheless, a clear relationship exists between the binding of hsp90 and altered kinase structure. We are currently utilizing the system described herein to investigate the role of hsp90 in cellular kinase folding and to investigate the specific sequences of $p56^{lck}$ whose structure is altered in conjunction with hsp90 binding.

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