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Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90

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ABSTRACT **Although Hsp90 displays general chaperone activity *in vitro*, few substrates of the chaperone have been identified *in vivo*, and the characteristics that render these substrates dependent on Hsp90 remain elusive. To investigate this issue, we exploited a paradoxical observation: several unrelated oncogenic viral tyrosine kinases, including v-src, attain their native conformation after association with Hsp90, yet their nearly identical cellular homologs interact only weakly with the chaperone. It has been controversial whether Hsp90 is vital for normal maturation of the cellular kinases or is simply binding a misfolded subfraction of the proteins. By modulating Hsp90 levels in *Saccharomyces cerevisiae*, we determined that Hsp90 is indeed necessary for the maturation of c-src (the normal homolog of v-src). c-src maturation is, however, less sensitive to Hsp90 perturbations than is v-src maturation. Dependence of the two proteins on Hsp90 does not correspond to their relative efficiency in reaching their final destination (the plasma membrane); we observed that in yeast, unlike in vertebrate cells, neither c-src nor v-src concentrate in the membrane. Expression of different v/c-src chimeras in cells carrying wild-type or temperature-sensitive Hsp90 alleles revealed that the difference between the proteins instead arises from multiple, naturally occurring mutations in the C-terminal region of v-src.**

The molecular chaperone Hsp90 is highly conserved throughout the eukaryotic lineage (1). In both organisms in which deletion mutants have been examined (*Saccharomyces cerevisiae* and *Drosophila melanogaster*), this ancient protein has proven essential for life (2, 3). Unlike other chaperones, Hsp90 appears to interact with substrates in their final stages of folding (4, 5). Even more unusual for a chaperone, particularly one that is among the most abundant cellular proteins, Hsp90 appears to have a rather limited range of target proteins *in vivo* (6). Known substrates, first identified by virtue of forming stoichiometric complexes with the chaperone, consist primarily of proteins involved in signal transduction. They include basic helix–loop–helix transcription factors, steroid hormone receptors, cellular serine/threonine kinases, and viral tyrosine kinases (6). Oddly, Hsp90 displays little or no detectable interaction with proteins closely related to these proteins (7). Examination of such proteins, nearly identical in sequence yet dramatically different in their associations with Hsp90, could therefore help to illuminate a fundamental engima of this chaperone—the features that govern its recognition of substrates.

One such pair of proteins, the nonreceptor tyrosine kinases v-src and c-src, form the focus of our study. v-src is the oncogenic protein encoded by the Rous sarcoma virus; c-src, the cellular counterpart from which it is derived, is involved in control of cell growth and cellular architecture (8, 9). v-src has a much higher kinase activity than c-src and differs from its homolog by eight amino acid substitutions and a small C-terminal replacement (10). Newly synthesized v-src in vertebrate cells can be coimmunoprecipitated with Hsp90 (11–15). Initial reports, however, indicated no such association between Hsp90 and c-src (16). Subsequently, other groups observed a weak interaction in that a relatively small percentage of c-src can be coimmunoprecipitated with Hsp90 (11, 17). Although Hsp90 is essential for formation of functional v-src, the significance of the interaction between the chaperone and c-src remains controversial. This same pattern of association with Hsp90 also pertains to several other oncogenic nonreceptor tyrosine kinases, including v-yes and v-fps and their cellular homologs (11, 12, 14). Is the interaction with Hsp90 necessary for maturation of the cellular proteins or does it simply represent a salvage or degradation pathway for a small fraction of the cellular proteins that fail to fold properly?

A case can be made in favor of either hypothesis. On one hand, it seems unlikely that the viral forms of src, yes, and fps all interact with Hsp90, while none of their cellular progenitors do; the viral proteins are far more similar to their cellular forms than to one another. Moreover, recent work demonstrating general chaperone activity of Hsp90 *in vitro* holds out the possibility that the range of Hsp90 substrates *in vivo* may extend beyond those with associations readily detectable by methods such as immunoprecipitation (18–25). On the other hand, because Hsp90 is a chaperone, its interactions with target proteins likely are determined by their state of folding. Unrelated mutations in all of these viral kinases might have produced conformations at once less tightly folded and less repressed than that of their cellular forms. The common feature of these viral kinases thus may be mutations rendering them less stable and therefore dependent on Hsp90.

We investigated this issue by examining the role of Hsp90 in the formation of functional c-src in comparison to v-src. To do so, we expressed the proteins in *S. cerevisiae*. Yeast cells provide an excellent model system for these studies for several reasons. First, there is no homolog of *src* in yeast (ref. 26, and confirmed by a search of the newly completed yeast genome sequence, available at the World Wide Web site http://genome-www.stanford.edu), whose activity could complicate interpretation of results obtained with the exogenous protein. Second, expression of *src* genes in yeast produces functional kinases comparable in activity to those produced in higher eukaryotes (10, 27, 28). Third, *S. cerevisiae* has low levels of any tyrosine kinase activity (29) and a very low background of tyrosine-phosphorylated proteins, facilitating measurement of src kinase activity (30). Fourth, not only Hsp90, but also the accessory chaperones with which it acts, are conserved from yeast to mammals (29, 31–35). Fifth, yeast cells have an advantage over vertebrate cell lysates (a commonly used

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Abbreviation: Csk, c-src kinase.

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system for Hsp90 studies) in that they provide the macromolecular crowding of a bona fide *in vivo* cytosol, an important consideration in chaperone studies. Finally, Hsp90 functions in *S. cerevisiae* can be modulated by convenient genetic manipulations.

By using this system, we reported previously that certain yeast mutations that reduce by approximately 90% the cellular concentration of Hsp90 virtually eliminate the activity of v-src but decrease only slightly that of c-src (30). The minor effect of Hsp90 mutations on c-src can be interpreted in two very different ways. It may be that Hsp90 normally acts in c-src maturation but more efficiently than in v-src maturation, such that little Hsp90 is required. Alternatively, only a small fraction of c-src molecules, perhaps those that have misfolded, require the chaperone. Two new *S. cerevisiae* strains with even greater reductions of Hsp90 levels now permit us to distinguish between these possibilities. One strain exclusively expresses the temperature-sensitive *hsp90*^{G170D} allele, in which the normal glycine residue at position 170 is replaced by aspartic acid; this mutation results in abrupt loss of Hsp90 function when cells are shifted to the restrictive temperature (36). In the other strain, the sole source of Hsp90 is a gene with the wild-type coding sequence but a mutated promoter, resulting in one-twentieth the normal level of wild-type Hsp90. We discovered that interaction between Hsp90 and c-src is indeed crucial for c-src maturation. Further investigation revealed that unlike in vertebrate cells, neither c-src nor v-src concentrate at the plasma membrane in yeast, indicating that their variation in Hsp90 dependence is intrinsic rather than the result of one protein being stabilized by more efficient insertion into the membrane. We then mapped to the C-terminal region of v-src the features responsible for the differences between it and c-src in their association with Hsp90. Together with continuing advances in the structural analysis of src kinases and Hsp90, our findings should help to finally elucidate the properties governing the interaction of Hsp90 with its substrates.

MATERIALS AND METHODS

Plasmid and Strain Construction. *Csk* expression vectors, as well as the following c/v-*src* chimeras, were the generous gift of David Morgan (University of California, San Francisco): Y316v-*src*, Y424c-*src*, Y424c-*src*M, Y314cv-*src*NM, Y314vc-*src*MC, Y314cvc-*src*MS, and Ha*csk* (10). Y426c/v-*src*SC was constructed by replacing sequence 5′ of the *Sph*I site in v-*src* with the equivalent fragment of c-*src* (10). The *src* genes are regulated by the galactose-inducible promoter *GAL1* in all of these vectors. The HMEKc-*src*M construct, in which c-src^{K295M} is regulated by the *MET3* promoter, was created by inserting the *Eco*RI/*Cla*I fragment of c-*src*M into the *Eco*RI/*Cla*I site of HMEK. HMEK was constructed by inserting a PCR fragment containing the *MET3* promoter into the *Eco*RI and *Bam*HI sites of pRS313 (E. C. Schirmer and S.L., unpublished data).

All yeast strains used were derivatives of W303. Yeast cells were transformed by using a Gene Pulser electroporator (Bio-Rad). Bacterial cells were transformed by using the calcium chloride method (37). In the G170D strain, both *HSC82* and *HSP82* are disrupted; the temperature-sensitive *hsp90*^{G170D} mutant allele, derived from the *HSP82* gene, is the only source of Hsp90. The *hsp90*^{G170D} gene is on a centromeric vector and is under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (36). *HSC82* and *HSP82* both are disrupted in the ILEP1 strain; Hsp90 is provided by the integrated *hsp82* mutant allele *ilep1*, which contains a partially deleted promoter but an intact coding region (J. Taulien and S.L., unpublished data).

Yeast Media. Cells were grown either in the rich medium YPDA (2% glucose/1% yeast extract/2% Difco Bacto Peptone/10 mg/ml adenine sulfate), or, when nutritional selection was required to maintain plasmids, in the minimal medium SD, SRaf, or SGal (2% dextrose, raffinose, or galactose, respectively; 0.67% yeast nitrogen base without amino acids, supplemented with essential amino acids and nucleotides). Solid media also contained 2% Bacto agar (Difco).

Induction of v-src, c-rc, v-abl, and Csk Expression. Galactose-regulated genes were induced by growing cells to early- or mid-logarithmic phase in SRaf lacking uracil, and then centrifuging, transferring to SGal lacking uracil, and incubating the culture for 5–7 hr. Induction of methionine-regulated genes was carried out by first growing cells to mid-logarithmic phase in minimal medium lacking histidine. Cells were then collected by centrifugation, washed five times with sterile distilled water, and transferred to minimal medium lacking both histidine and methionine. In experiments that used the Hsp90^{G170D} temperature-sensitive mutant, 32°C and 34°C were used as the semirestrictive and restrictive temperatures, respectively (36).

Immunoblot Analysis, Quantitation, and Immunofluorescence. Cellular proteins were extracted, separated, blotted, and probed as described (36). Blots were stained with Coomassie blue to confirm equal sample loading. Antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) was used to detect phosphotyrosine residues, and antibody LA074 (Quality Biotech, Camden, NJ) was used for detection of v-src or c-src. Immune complexes were visualized by using enhanced chemiluminescence (Amersham). For quantitation, the same procedure was followed except that ^{125}I-conjugated Protein A was employed, and the blots were analyzed by using a PhosphorImager (Molecular Dynamics). Data was normalized to the total protein content in each lane, which was determined by using densitometric analysis of Coomassie-blue stained blots (Molecular Dynamics). Background levels of tyrosine phosphorylation in isogenic untransformed yeast strains were subtracted from each sample. At least three independent experiments were performed for each quantitation. Immunofluorescence was carried out as described (38) using a fluorescein-labeled secondary antibody for detection.

RESULTS

Hsp90 Is Essential for Maturation of c-src as a Tyrosine Kinase. We first examined the synthesis and activity of c-src in *S. cerevisiae* expressing the temperature-sensitive Hsp90-mutant protein G170D, which rapidly loses function when cells are shifted from 25°C to 34°C—the restrictive temperature for the mutant (36). Under the conditions employed, the mutant cells were fully viable, their rate of protein synthesis was unaffected, and cellular proteins remained soluble and functional (36, 39). Wild-type and G170D cells were transformed with the avian c-*src* gene driven by a galactose-inducible promoter. c-src expression was induced at the same time that cells were transferred to 34°C. Both strains produced a similar amount of c-src, as determined by reacting electrophoretically separated total proteins with an antiserum that detects both c-src and v-src (Fig. 1*A*). We then examined levels of tyrosine phosphorylation mediated by c-src. Probing total cellular proteins with an antibody recognizing phosphorylated tyrosine in polypeptides revealed that the tyrosine kinase activity of c-src was much lower in the mutant than in the wild-type cells (Fig. 1*B*). We quantified both of these results by using ^{125}I-Protein A. In the G170D mutant, c-src accumulated to 80% of the level present in wild-type cells at 34°C, but the tyrosine kinase activity of c-src in G170D was only ≈20% that of wild-type cells (data not shown). Thus, under these conditions, Hsp90 is required for full c-src tyrosine kinase activity.

We were concerned, however, that high temperatures may in some manner affect the general chaperone machinery of yeast or alter the conformation of c-src, thus rendering the kinase artifactually dependent on Hsp90. We therefore examined the

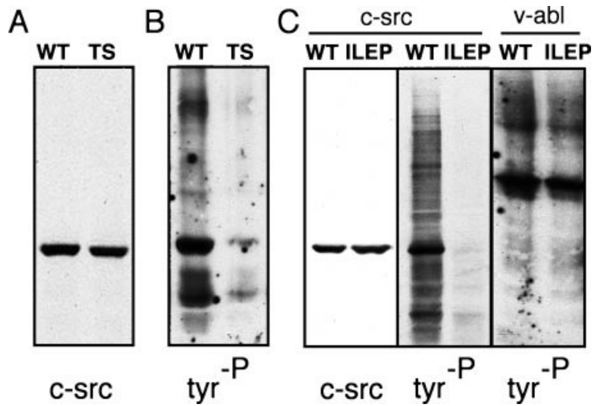

FIG. 1. Effects of Hsp90 mutant strains on c-src and v-abl. (*A*) Accumulation of c-src protein in wild type (WT) and G170D mutant (TS) cells at 34°C. (*B*) c-src-mediated tyrosine phosphorylation in the two strains. Cells were grown to early logarithmic phase in SRaf at 25°C then transferred to SGal at 34°C for 5 hr. (*C*) From *Left* to *Right*, accumulation of c-src in wild-type (WT) and ILEP cells; c-src-mediated tyrosine phosphorylation in wild-type and ILEP strains; and v-abl-mediated tyrosine phosphorylation in wild-type and ILEP1 cells.

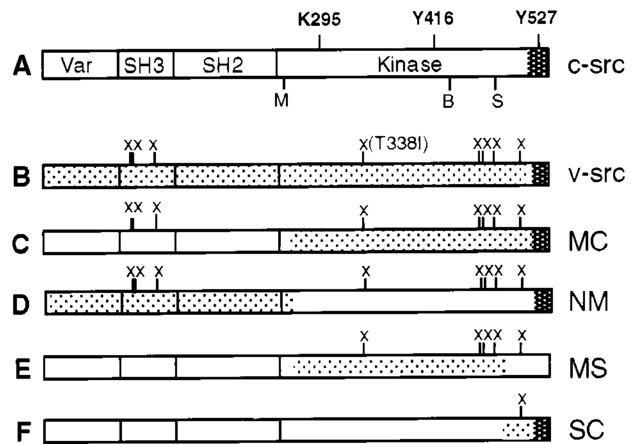

FIG. 2. src proteins used in this work. (*A*) Diagram of c-src, indicating the boundaries of the variable (Var), SH3, SH2, and kinase domains. Also shown are the location of the negative regulatory phosphorylation site (Y527), the major autophosphorylation site (Y416), and the conserved lysine in the ATP binding site (K295). M, B, and S, *Mlu*I, *Bgl*I, and *Sph*I restriction sites, respectively, used to make v-*src*/c-*src* chimeras. (*B*) Diagram of v-src, indicating the amino acid changes (marked by X) and the C-terminal sequence (dark shading) that differ from c-src (see text). (*C–F*) v-src/c-src chimeras. Shaded portions represent sequences derived from v-src. The c-src/v-src chimeras are named according to the v-*src* restriction fragments they contain (e. g., MS is a chimera in which a *MluI-Sph*I fragment of v-src is flanked by c-src sequences) (10).

dependence of c-src tyrosine kinase activity on Hsp90 at normal temperature (25°C), taking advantage of another Hsp90 mutant, ILEP1. Both *HSC82* and *HSP82* are deleted in ILEP1; the only source of Hsp90 is an integrated *hsp82* allele encoding wild-type protein but carrying a partial deletion of the promoter, resulting in 1/20 the normal levels of Hsp90 expression. Although the cells are very temperature-sensitive, they grow at near-wild-type rates in minimal media at 25°C (J. Taulien and S.L., unpublished data).

In the ILEP1 strain at 25°C, c-src accumulation was not affected, but the kinase activity of c-src was greatly reduced (Fig. 1*C*). Quantitation of immune complexes showed that despite nearly equal levels of c-src protein, tyrosine phosphorylation by c-src in the ILEP1 cells was ≈20% of that in wild-type cells (data not shown). Thus, loss of kinase activity is not simply the result of decreased c-src stability. The reduction of activity in ILEP1 cells at 25°C was as significant as in G170D cells at 34°C, providing independent evidence that maturation of c-src requires Hsp90.

To ascertain whether the effect of Hsp90 on c-src is specific rather than perhaps a general indirect effect on tyrosine phosphatases, we examined the maturation of another nonreceptor tyrosine kinase, v-abl. The v-*abl* gene, which was identified from the Abelson murine leukemia virus, does not belong to the *src* family (26, 40, 41), and there is no evidence that v-abl interacts with Hsp90. Because it is unstable in yeast cells at 34°C, v-abl cannot serve as a control in experiments utilizing the G170D strain (data not shown). At 25°C, however, this kinase is stable and functional (30), allowing us to use the ILEP1 strain to examine the effects of reduced Hsp90 levels on the activity of v-abl. Tyrosine phosphorylation mediated by v-abl was not affected in the ILEP1 strain relative to wild-type cells (Fig. 1*C*), demonstrating that the dependence of c-src on Hsp90 is indeed specific, as previously determined for v-src (30).

Maturation of c-src as a Substrate of the Regulatory Kinase Csk Also Requires Hsp90. As a second, independent assay of c-src maturation, we asked whether Hsp90 is required for c-src to attain a conformation that can be recognized by its regulator Csk (c-src kinase). Csk suppresses c-src kinase activity by phosphorylating the protein on Tyr-527 (Fig. 2). Csk expressed in yeast cells is functional as well as specific: when c-src and Csk are coexpressed in yeast, c-src is the only protein phosphorylated by Csk (10).

To determine whether Hsp90 is required to facilitate maturation of c-src as a substrate of Csk, we coexpressed c-*src* and *csk* in wild-type and in G170D cells. For this experiment, we used a mutant of c-src containing a single amino acid substitution in the kinase domain (lysine to methionine in residue 295), which eliminates kinase activity (Fig. 2). Use of this protein, which has the same capacity as wild-type c-src to serve as a substrate for Csk (10), ensured that any tyrosine phosphorylation of c-src resulted solely from the activity of Csk on Tyr-527 rather than from autophosphorylation by c-src.

Because Csk is somewhat unstable at 34°C in yeast (data not shown), we used a temperature which is semirestrictive for G170D (32°C). It was first necessary to confirm that Csk activity is not itself affected by the G170D mutation under these conditions. To test that possibility, *c-src*^{K295M} was placed under the control of the *MET3* promoter, which is induced in media lacking methionine and repressed when methionine is present. *csk* expression was driven by the *GAL1* promoter and therefore was induced by galactose. The two constructs were cotransformed into wild-type and G170D strains. c-src^{K295M} was allowed to accumulate under normal maturation conditions by growing wild-type and G170D cells overnight at 25°C in minimal raffinose medium lacking methionine. Production of c-src^{K295M} was then repressed, and that of Csk induced, by transferring cells to galactose medium containing methionine. At the same time, the temperature was increased to 32°C to reduce Hsp90 function in the G170D strain. Wild-type cells and the G170D mutant displayed equal levels of c-src^{K295M} and Csk (Fig. 3*A*). Moreover, c-src phosphorylation was equivalent in both strains (Fig. 3*A*). Thus, the G170D mutation does not influence Csk accumulation or activity at 32°C.

We could therefore use these cells to investigate whether Hsp90 is required for the maturation of c-src as a substrate of Csk. Cells were grown overnight at 25°C in minimal raffinose medium containing methionine then transferred to 32°C in galactose medium lacking methionine to induce expression of c-src^{K295M} and Csk (Fig. 3*B*). Accumulation of c-src^{K295M} and Csk was only slightly decreased in the G170D cells, but phosphorylation of c-src^{K295M} by Csk was significantly reduced in that strain relative to wild-type (Fig. 3*B*). Quantitation of immune complexes indicated that c-src^{K295M} phosphorylation

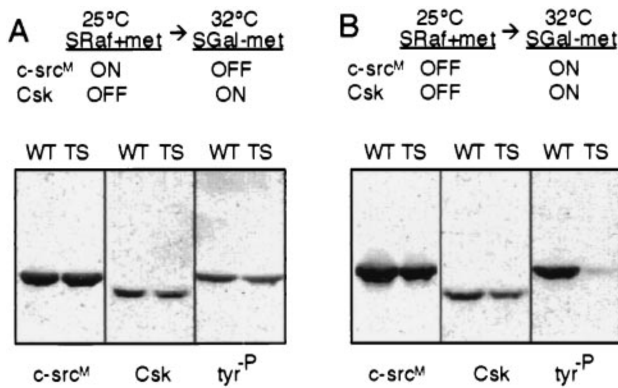

FIG. 3. Effect of the temperature-sensitive mutation *hsp90*^{G17OD} on the activity of the c-src regulatory kinase Csk and on the maturation of c-src^{K295M} at the semirestrictive temperature. (*A*) Csk produced at 32°C: accumulation of c-src^{K295M} and Csk and Csk-mediated tyrosine phosphorylation of c-src^{K295M} in wild-type (WT) and G170D (TS) cells. (*B*) Csk and c-src^{K295M} produced at 32°C: accumulation of c-src^{K295M} and Csk and Csk-mediated tyrosine phosphorylation of c-src^{K295M} in wild-type (WT) and G170D (TS) cells.

in the mutant cells was ≈20% that in wild-type for nearly equal levels of c-src^{K295M} and Csk (data not shown). Because the prior experiment demonstrated that Csk activity in G170D mutant cells is equivalent to that of wild-type cells at 32°C, the reduced phosphorylation must be caused primarily by a deficiency in the maturation of the substrate of Csk, c-src^{K295M}, when Hsp90 function is reduced. These results demonstrate that Hsp90 is crucial not only for the maturation of c-src as a kinase but also for its maturation as a substrate of the regulatory tyrosine kinase Csk.

Neither c-src nor v-src Localizes to the Plasma Membrane in Yeast. Although the previous experiments demonstrate that the maturation of c-src depends on Hsp90, earlier work showed that c-src is less affected than v-src by perturbations in Hsp90 levels (30). We considered that this difference could arise if c-src were more efficient than the viral protein in reaching its ultimate destination, the plasma membrane. Insertion into the membrane might stabilize c-src and thereby diminish its need for Hsp90.

Although we cannot eliminate this possibility, the results of immunofluorescence staining of wild-type *S. cerevisiae* cells expressing either protein, however, make it seem unlikely. Both c-src and v-src were distributed evenly in the cells, indicating that unlike in vertebrate cells, neither protein is efficiently targeted in yeast (Fig. 4). In contrast, a GST–ras fusion protein did localize to the plasma membrane as expected (ref. 42; Fig. 4). Similar conclusions were drawn from Western blot analysis of membranes in fractionated cell lysates (data not shown).

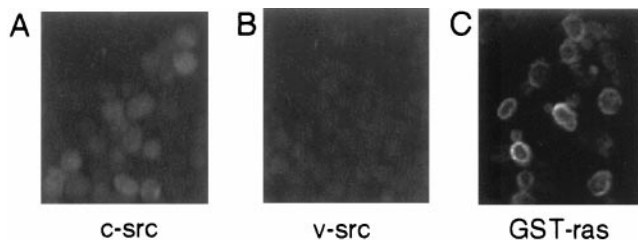

FIG. 4. Immunofluorescece analysis of c-src and v-src distribution in wild-type yeast cells. Intracellular distribution of c-src (*A*), v-src (*B*), and a GST-ras fusion protein (*C*). Cells were grown in raffinose medium to early logarithmic phase then transferred to galactose-containing medium for 5 hr to induce expression of the indicated protein. As expected from its lower level of accumulation, the fluorescence signal from v-src was lower than that of c-src but well above background (staining with secondary antibody in the absence of primary antibody), which was not detectable at this level of exposure (not shown).

Multiple Mutations in the C-Terminal Region of v-src Determine Its High Dependence on Hsp90. The observations described above suggested that the variation in Hsp90 dependence between c-src and v-src results from differences intrinsic to the two proteins. We therefore attempted to identify the specific modifications in v-src that contribute to its increased need for Hsp90. As noted earlier, v-src contains eight single amino acid substitutions, as well as a short C-terminal replacement not present in c-src. We expressed chimeras of v-*src* and c-*src* and compared their activity and accumulation in wild-type and G170D cells at 32°C. This semirestrictive temperature was chosen because it produces a much stronger effect on v-src than on c-src, thereby providing a range over which to judge the relative sensitivities of the chimeric constructs.

Schematic diagrams of the various proteins tested are shown in Fig. 2. Among them, v-src, NM, MC, and MS were expressed from single copy, centromeric vectors; multiple-copy vectors were used for c-src and SC to increase the phosphorylation signal obtained from these relatively weak kinases. As expected from the Csk experiment, c-src activity was reduced somewhat in the G170D mutant (Fig. 5 *A* and *C*). Note that this property of c-src was less sensitive to decreased levels of Hsp90 than was the maturation of c-src as a substrate for Csk: the kinase activity of c-src in G170D cells was reduced to about 75% that in wild-type cells, whereas the ability of c-src to serve as a substrate of Csk was reduced to 20% that in wild-type cells under the same conditions (Fig. 3*B*, and data not shown). In any event, v-src activity decreased far more dramatically (Fig. 5 *A* and *C*). Moreover, v-src protein levels decreased, but c-src levels were unaffected (Fig. 5*B*). These results confirm in this strain the observations reported with other strains that v-src kinase activity is more dependent on Hsp90 than is c-src activity.

The activities of the various src chimeras were concurrently examined in wild-type and G170D cells exposed to 32°. Tyrosine phosphorylation mediated by these chimeras was reduced to different extents (Fig. 5 *A* and *C*), suggesting that the maturation of the individual chimeras had different degrees of dependence on Hsp90.

The src chimeras NM and MC are reciprocal constructs: NM encodes the N-terminal half of v-src and C-terminal half of c-src, and MC the reverse (Fig. 2). The tyrosine kinase activity of NM was only slightly affected in the G170D mutant—activity remained ≈70% of NM activity in wild-type cells, similar to the change in activity observed for c-src (Fig. 5 *A* and *C*). The tyrosine kinase activity of MC, however, was very much dependent on Hsp90 function—activity declined to ≈25% of MC activity in wild-type cells, a decrease close to that observed with v-src (Fig. 5 *A* and *C*). These results indicate that mutations in the C-terminal segment of v-src play a key role in the greater sensitivity of that protein to reduction in Hsp90 function. This observation fits well with reports by others that it is this region of v-src which physically associates with Hsp90 (11). Interestingly, the MC protein accumulated to a lower level than NM (Fig. 5*B*); unfortunately, neither MC nor v-src protein levels were high enough for quantitation.

To further dissect the importance of the C-terminal mutations in the interaction of v-src with Hsp90, two other chimeras, MS and SC, were analyzed. The N-terminal regions of both are derived from c-src, but the structures of their C-terminal portions are reciprocal: MS has the *Mlu*I–*Sph*I fragment from v-*src*, whereas SC has the *Mlu*I–*Sph*I fragment from c-*src* (Fig. 2). The dependence of MS and of SC on Hsp90 (Fig. 5 *A* and *C*) showed no significant differences and were very similar to that of c-src, indicating that the *Mlu*I–*Sph*I and the *Sph*I-3′-end fragments both carry mutations that, although necessary, are not sufficient for the high Hsp90 dependence of v-src. More-

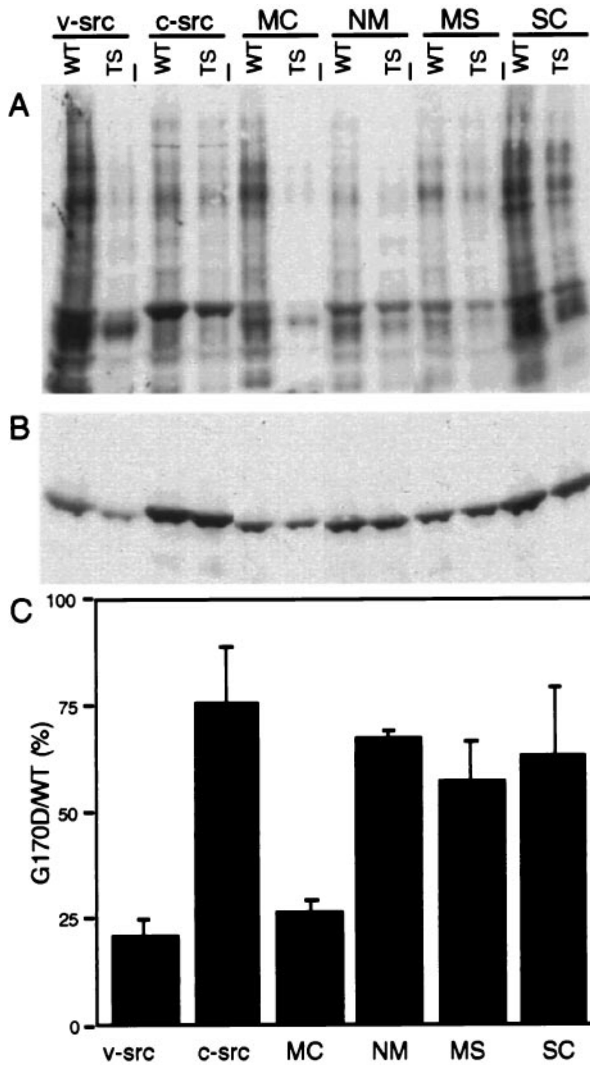

FIG. 5. Regions of v-src responsible for increased dependence on Hsp90. Tyrosine phosphorylation mediated by src kinases (*A*) and accumulation of src proteins in wild-type (WT) and G170D (TS) strains at the semirestrictive temperature (*B*). See Fig. 2 and text for description of the src chimeras.

over, the Y527F mutant of c-src, which is permanently activated, depends on Hsp90 to an extent similar to that of c-src (data not shown). We conclude that multiple modifications in the C-terminal region of v-src are responsible for its increased dependence on Hsp90.

DISCUSSION

We have shown that the molecular chaperone Hsp90 is required for the maturation of c-src both as a kinase and as a substrate of the regulatory kinase Csk. Earlier work had shown that c-src activity is less perturbed than that of v-src by decreased levels of Hsp90. This difference seems unrelated to the insertion of the proteins into the plasma membrane; when expressed heterologously in yeast, neither protein concentrates in the membrane. Instead, we found that their variation in Hsp90 dependence is intrinsic to the proteins and results from multiple differences in their C-terminal region. Our results bear on several fundamental issues regarding Hsp90: its range of substrates *in vivo*, the manner in which it interacts with its substrates, and the features it recognizes in those substrates.

Hsp90 was originally thought to play a critical role in the functioning of only a small number of proteins in the cell. Our observations resolve the long-standing controversy surrounding the significance of the interaction between Hsp90 and c-src, and in doing so, extend the known role of Hsp90 in signal transduction from processes involving endogenous serine/threonine kinases and exogenous tyrosine kinases to include those processes mediated by normal cellular tyrosine kinases. Similarly, Holley and Yamamoto (7) determined that nonsteroid hormone nuclear receptors, which had not been detected in stable complexes with Hsp90, nevertheless were dependent on the chaperone for maturation. Their work also used *S. cerevisiae* strains engineered to have reduced levels of Hsp90. Although it is formally possible that our observations and theirs are artifacts of the yeast system, this prospect is unlikely. Protein-folding requirements are universal, as are chaperone systems in general. Those systems, and as noted earlier, Hsp90 and its cochaperones in particular, are very highly conserved in eukaryotic cells. Remarkably, human Hsp90 can substitute for the essential functions of yeast Hsp90 *in vivo* (43, 44), and recent assays demonstrate a striking similarity in the chaperone activities of the two proteins *in vitro* (21, 45).

Our observations also clarify the results of previous experiments with vertebrate cells, in which it was not possible to distinguish whether interaction with Hsp90 is required for kinase maturation or for membrane targeting; a central role in trafficking had been hypothesized (46). It now appears from our studies that Hsp90 is necessary for the maturation of src kinases *per se*. The inefficient targeting of src proteins to the plasma membrane in yeast suggests these cells would provide an excellent environment in which to identify vertebrate factors responsible for insertion of src proteins into the membrane. More importantly, this observation establishes *S. cerevisiae* as an ideal system for analyzing factors specific to the folding and maturation of src kinases without having to distinguish such factors from those involved in membrane targeting.

Our finding that the distinction in Hsp90 dependence between c-src and v-src maps to multiple substitutions in the latter's C-terminal domain is consistent with previous work in vertebrate cells that demonstrated that the C-terminal region is necessary and sufficient for physical association with Hsp90 (11). That region includes the tyrosine kinase domain, the activity of which is increased in v-src by the mutations and the small replacement at the C terminus (47). Recent structural and functional work, however, indicates that residues in the SH3 domain (located in the N-terminal half of the protein) that are mutated in v-src also are important for the elevated activity of the viral protein. The crystal structure of c-src shows that these amino acids make specific contacts with the catalytic domain of the protein; mutation of these sites alone in c-src relieves the repression caused by interaction of the SH3 and catalytic domains, enabling the protein to become partially activated (48–50). Additional work on c-src and v-src, as well as on Hsp90, is needed to fully elucidate how the various regions of the kinases contribute to activity and Hsp90 dependence, as well as the relationship of these two properties.

We propose that the critical difference between the two kinases in their dependence on Hsp90 stems from differences in the folding of the proteins. These differences may cause v-src to require greater interaction with Hsp90 for maturation and stability. Of the various proteins we tested, only two—v-src itself and the MC chimera—were highly sensitive to Hsp90 perturbations. They also were the only two that had difficulty accumulating, a characteristic of proteins with unstable structures. Such differences in stability also may underlie the differing requirements for Hsp90-associated accessory chaperones seen with v-src and c-src. Hsp90 and Cdc37 (p50), both of which have been coimmunoprecipitated with v-src and c-src (11), may form a core chaperone machinery for folding the two proteins. Three additional Hsp90-associated chaperones, Sti1,

Cpr6, and Cpr7, are needed for maturation of v-src but not of c-src (33, 51). Underscoring the central role of Hsp90 and Cdc37 is the observation that both proteins are essential in *S. cerevisiae*, whereas Sti1, Cpr6, and Cpr7 are not (33, 51). It will be interesting to test whether Cdc37 is crucial for c-src maturation. The *S. cerevisiae* system again provides an excellent opportunity for such study.

Together with the results reported here, our hypothesis that the folding state of a substrate determines the degree to which its maturation requires Hsp90 provides a satisfying explanation for the puzzling observations discussed earlier that viral kinases such as src, yes, and fps both are more active than their cellular homologs and are readily immunoprecipitated with Hsp90, whereas their cellular forms are not (11, 12, 14). As we have demonstrated for c-src, even transient or weak interactions between Hsp90 and certain target proteins may be vital for the proper folding of those proteins. It therefore is likely that these other cellular tyrosine kinases also will prove to require Hsp90 for maturation. More generally, however, the nature of Hsp90–substrate interactions are probably determined less by convergence in the sequence of substrates than by their state of folding. Unrelated mutations in all of these viral kinases may produce less tightly folded conformations than are present in their cellular counterparts. That, in turn, could make the viral proteins simultaneously less repressed and more dependent on Hsp90. This principle, and the findings that give rise to it, point to a much broader role in crucial signal-transduction processes for this ubiquitous and enigmatic chaperone.

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