MOLECULAR AND CELLULAR BIOLOGY, Nov. 1992, p. 5059–5068
0270-7306/92/115059-10$02.00/0

Vol. 12, No. 11

Conformational Activation of a Basic Helix-Loop-Helix Protein (MyoD1) by the C-Terminal Region of Murine HSP90 (HSP84)

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Received 29 June 1992/Returned for modification 23 July 1992/Accepted 24 August 1992

A murine cardiac λgt11 expression library was screened with an amphipathic helix antibody, and a recombinant representing the C-terminal 194 residues of murine HSP90 (HSP84) was cloned. Both recombinant and native HSP90s were then found to rapidly convert a basic helix-loop-helix protein (MyoD1) from an inactive to an active conformation, as assayed by sequence-specific DNA binding. The conversion process involves a transient interaction between HSP90 and MyoD1 and does not result in the formation of a stable tertiary complex. Conversion does not require ATP and occurs stoichiometrically in a dose-dependent fashion. HSP90 is an abundant, ubiquitous, and highly conserved protein present in most eukaryotic cells. These results provide direct evidence that HSP90 can affect the conformational structure of a DNA-binding protein.

Heat shock proteins (HSPs) are thought to aid cells coping with stress, and their expression is induced by heat, heavy metals, pH, and alcohol (reviewed in references 13 and 16). Recent studies have shown that some classes of HSP (HSP60 and HSP70) also function in membrane translocation and folding of proteins in cells that are not under stress (reviewed in reference 13). Synthesis of HSP90 increases in some cells three to fivefold under stress conditions, but it is also abundant in uninduced cells (22). Most of the information acquired so far about the function of HSP90 has been through association, and an activity for the protein has not been described in vitro. HSP90 interacts with several viral oncogene products that display tyrosine kinase activity (4, 25, 31, 39), associates with tubulin and actin (28, 36), stimulates the activity of eukarytotic initiation factor 2 α subunit-specific protein kinase (33), and forms stable complexes with some members of the nuclear receptor superfamily (see below). Sequence analysis has shown that HSP90 (the generic name for this family used here) is highly conserved among species (summarized in reference 15): human (HSP90), mouse (HSP84), chicken (HSP90), *Drosophila* (HSP83), *Trypanosoma* (HSP85), and yeast (HSP83) homologs display a minimum of 65% amino acid conservation.

Members of the nuclear receptor superfamily display DNA-binding activity and translocate from the cytoplasm to the nucleus after acquiring a ligand. The inactive cytoplasmic forms of the glucocorticoid (18 [and references within], 35), estrogen (7 [and references within]), progesterone (6), and aryl hydrocarbon (AH) (29) receptors exist as stable complexes containing HSP90, whereas the ligand-activated nuclear forms lack HSP90. HSP90 is thought to negatively regulate activation and translocation of the cytoplasmic forms of the receptors in the absence of ligand (1). Substantial evidence suggests that binding of HSP90 may be essential to the subsequent activity of these receptors and that HSP90 may be involved in their initial folding. Glucocorticoid receptor translated from a reticulocyte lysate, which contains HSP90, displayed high-affinity hormone-binding activity, whereas receptor translated from a wheat germ lysate, which lacks HSP90, did not express hormone-binding activity (9). In addition, glucocorticoid receptor expressed in strains of yeast containing 20-fold-lower levels of HSP90 displayed significantly less hormone-induced transcriptional activation in vivo than receptor expressed in yeast strains bearing normal levels of HSP90 (30).

The AH receptor also binds HSP90 in the cytoplasm and dissociates from it upon binding ligand (14, 29). Ligand-bound AH receptor translocates to the nucleus as a heterodimer with the AH receptor nuclear translocation protein, where it activates transcription of the cytochrome P-450 genes (12, 14, 17, 29). The AH receptor nuclear translocation protein has recently been shown to contain a basic helix-loop-helix (bHLH) consensus sequence, which is common to the dimerization and DNA-binding domains of a large group of proteins that includes Myc and MyoD1 (17, 27). bHLH proteins bind DNA as dimers. They are characterized by a dimerization motif consisting of two amphipathic α helices separated by nonhelical loop and a DNA-binding motif consisting of a stretch of basic amino acids immediately N terminal to the first amphipathic helix. Since the primary sequence of the AH receptor has not been determined, it is not yet known whether the receptor also contains a bHLH domain or whether dimerization with the AH receptor nuclear translocation protein is mediated by such a structure. Nonetheless, it is an intriguing possibility that HSP90 can regulate the dimerization of two bHLH proteins.

Until now, a direct demonstration of the function of HSP90 in protein folding and assembly has not been described. In this report, the DNA-binding activity of a bHLH protein (MyoD1) synthesized in *Escherichia coli* is shown to be significantly improved by treatment with either native murine HSP90 or a recombinant protein consisting of the C-terminal 194 amino acids of murine HSP90 fused to the maltose-binding protein (MBP) of the *malE* gene (referred to as MBP-HSP90). The bHLH domain of MyoD1 (a myogenic determination protein) mediates binding to a consensus sequence present in the enhancer elements of several genes

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whose expression is restricted to muscle (24; reviewed in reference 38). MyoD1 binds this sequence as either a homodimer or a heterodimer with products of the E2A gene (27). Activation of the homodimer, which occurs in the absence of exogenous ATP, results from a transient interaction between MBP-HSP90 and MyoD1 during which the conformation of MyoD1 is altered and the activation potential of MBP-HSP90 is depleted. The results presented here demonstrate in vitro a protein-folding activity of HSP90.

MATERIALS AND METHODS

Antibody generation. Synthetic peptide fragments representing murine MyoD1 residues 141 to 162 and murine c-Myc residues 387 to 408 were individually coupled to keyhole limpet hemocyanin by dissolving keyhole limpet hemocyanin plus peptide in water (500 μg/ml each) and dialyzing the mixture against 0.1% glutaraldehyde. Residual glutaraldehyde was removed by exhaustive dialysis in normal saline. Rabbits were immunized with 500 μg of total protein emulsified in complete Freund's adjuvant starting with either the MyoD1 or c-*myc* peptide and then 2 weeks later with the other peptide emulsified in incomplete Freund's adjuvant. Injections of either the MyoD1 or c-*myc* peptide were alternated every 2 weeks, and three cycles (six injections) were completed. A final boost was performed with both peptides, and serum was collected 2 weeks later.

Antibodies were purified from the pooled sera by affinity chromatography. Peptides were coupled to cyanogen bromide-activated Sepharose 4B by using a protocol provided by the manufacturer (Pharmacia). All column procedures were performed at 4°C. The serum was run through the column, and bound antibody was washed with several volumes of Tris-buffered saline (TBS; 20 mM Tris, pH 7.4; 150 mM NaCl). Bound antibody was then washed with TBS supplemented with 0.5 M NaCl. Antibodies first were eluted with 100 mM glycine (pH 2.7)–150 mM NaCl and then eluted with 50% ethylene glycol in 5 mM lysine. The fractions were dialyzed exhaustively against TBS and then pooled and concentrated with Centricon devices (Amicon).

Cloning, sequencing, and protein expression. A murine heart λgt11 library was purchased from Clontech. Approximately 10^7 independent phage clones were screened with the amphipathic helix antibody. Standard procedures for plating the phage, inducing fusion protein expression, and absorbing proteins to nitrocellulose were used (19). Antibody screening was performed after the nitrocellulose sheets were fixed with 10% acetic acid–25% isopropanol and then washed at least 12 h with several changes of water. The sheets were blocked with 5% nonfat dry milk in TBS (4 h, 4°C) and then incubated with antibody (5 μg/ml) in TBS supplemented with 0.5 M NaCl, 5% nonfat dry milk, 5% horse serum, and 0.5% Tween 20 (18 h, 4°C). The sheets were washed twice with TBS supplemented with 0.5% Tween 20 and then incubated (18 h, 4°C) with 50 nCi of ^{125}I-anti rabbit immunoglobulin G per ml (using the same buffer composition as was used for primary antibody). The sheets were washed several times with TBS supplemented with 0.5 M NaCl and 0.5% Tween 20 and then autoradiographed by using Kodak XAR-5 film and Dupont Lightning-Plus intensifying screens.

Positive clones were recovered as plugs and rescreened twice (to homogeneity), and λ phage DNA was then prepared according to standard protocols (34). The cDNA inserts were removed with *Eco*RI and ligated into pBluescript (Stratagene) for sequencing or pmal-cR1 (New England Biolabs) for protein expression. Single-stranded DNA was prepared for sequencing from pBluescript by using VCS-M13 noninterfering helper phage and standard protocols (34). Sequencing of single-stranded DNA was performed with Sequenase version 2.0 by using the protocols provided by the manufacturer (United States Biochemical). The pmal-cR1 *Eco*RI site is oriented in the same frame as the *lacZα Eco*RI site of λgt11, so that the cDNA insert was expressed without further modifications. Selected clones expressing MBP-HSP90 were validated by sequencing. Growth of transformed hosts, induction of protein expression, and affinity purification of the expressed proteins were performed as described in a manual produced by the manufacturer of the pmal-cR1 vector (New England Biolabs). MBP-HSP90 was eluted from the amylose column into buffer D (see below) containing 10 mM maltose.

Western blotting (immunoblotting) and protein analysis. Homogenates or purified proteins were dissolved in 100 mM Tris (pH 6.8)–2% sodium dodecyl sulfate (SDS)–0.25% phenol red–10% (vol/vol) glycerol–5% (vol/vol) β-mercaptoethanol and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) as described previously (21). Transfers to nitrocellulose were performed as described by the manufacturer of the apparatus used (Bio-Rad). Nitrocellulose sheets were fixed and blotted with amphipathic helix antibody as described above. Coomassie stains were performed on gels that had been fixed in 10% acetic acid–25% methanol by using a colloidal concentrate and the protocol provided by the manufacturer (Sigma).

Electrophoretic mobility shift assays (EMSAs). T7-MyoD1 was generated with the pRK171a vector and isolated from bacterial extracts by ion-exchange chromatography. This reagent was a gift from H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, Wash.). The Glu-MyoD1 deletion mutant in Fig. 10 is described in the work of Lassar et al. (24) and was a kind gift of A. Lassar and H. Weintraub (Fred Hutchinson Cancer Research Center). Complementary oligomers derived from the right MyoD1 binding site of the muscle creatine kinase enhancer (5) were end labelled with T4 polynucleotide kinase prior to hybridization. Gel shift assay conditions were similar to those described by Buskin and Hauschka (5). Incubations and gel electrophoresis were performed at 4°C (important). Final reaction volumes were equal in all comparisons and did not exceed 20 μl. Standard reaction conditions included 200 ng of MyoD1 and 50 ng of oligomers in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9)–10% glycerol–50 mM KCl–0.2 mM EDTA–0.5 mM dithiothreitol (buffer D), supplemented with 50 μg of poly(dI-dC) and 250 μg of acetylated bovine serum albumin (BSA) per ml. Complexes were resolved by 5% PAGE (30:1) buffered with 100 mM Tris, 50 mM boric acid, and 1 mM EDTA (pH 8.0). Samples were run at 200 V (0.75-mm gel thickness; 15-cm width) for 2 to 3 h. After electrophoresis gels were dried and autoradiographed with Kodak XAR-5 film.

For heat denaturation experiments, MyoD1 was diluted (1:5) into water, heated to 45°C for 15 min, cooled on ice, and brought to 1× buffer D. MBP-HSP90 is resistant to heat denaturation, requiring at least 70°C for 45 min for inactivation (in buffer D). No precipitated protein was observed after this treatment.

RESULTS

Expression cloning of a C-terminal fragment of murine HSP90 with an amphipathic helix antibody. A polyclonal antiserum was generated by immunizing rabbits with syn-

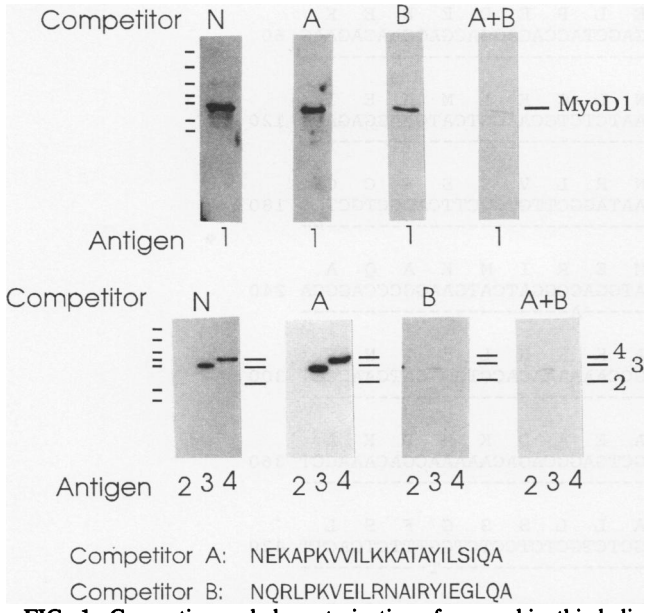

FIG. 1. Generation and characterization of an amphipathic helix antibody. Competitor A represents the second amphipathic helix of the murine c-Myc bHLH domain, and competitor B represents the second amphipathic helix of the murine MyoD1 bHLH domain. Rabbits were immunized at 2-week intervals with alternating doses of each peptide (competitor A or B) coupled to keyhole limpet hemocyanin, the sera were collected, and the amphipathic helix antibodies were affinity purified by using both peptides. The antibody was then characterized by Western blotting. Antigens: 1, recombinant MyoD1 protein (100 ng); 2, deletion mutant of MyoD1 lacking the HLH domain (100 ng of recombinant glutathione-*S*-transferase fusion protein); 3, recombinant glutathione-*S*-transferase myogenin fusion protein (100 ng); 4, recombinant MBP-HSP90 (100 ng; described in Fig. 3). Competitors (A and B) were preincubated with antibody overnight prior to Western blotting. N, no competitor. The antibody does not react with the MyoD1 deletion mutant lacking an HLH domain and cross-reacts with myogenin and MBP-HSP90. The peptide derived from MyoD1 was a more potent inhibitor.

thetic peptides representing the second amphipathic helices of the bHLH domains of murine c-Myc and MyoD1 (Myc residues 387 to 408; MyoD1 residues 141 to 162). Antibodies were affinity purified with the same peptides and then tested for specificity by Western blotting with recombinant MyoD1 protein. The affinity-purified antibody, which reacted with MyoD1 in Western blots, was slightly inhibited by preincubation with the *myc* peptide, partly inhibited by preincubation with the MyoD1 peptide, and completely inhibited by preincubation with both (Fig. 1, top). The antibody did not react with a deletion mutant of MyoD1 lacking residues 141 to 162 but did react with myogenin (which contains a homologous bHLH domain) and with a fusion protein containing the C-terminal 194 amino acids of murine HSP90 (described below; Fig. 1, bottom).

The amphipathic helix antibody was used to screen a murine heart λgt11 prokaryotic expression library for cross-reactive proteins, originally as a means of cloning structurally analogous transcriptional activators from myocardium. Several phage clones one of which contained an 806-base cDNA insert with 97% homology to the 3′ 820 bases of murine HSP90 (Fig. 2), were isolated. The clone (the cDNA insert was shuttled to pBluescript and is referred to as pBS33) encodes 194 amino acids bearing 95% homology to the C-terminal 194 amino acids (531 to 724) of murine HSP90. The nucleotide differences between the published sequence and this clone manifest in the following amino acid variations: six changes in the first 8 residues, a valine-to-phenylalanine change at residue 45, and a valine-to-glycine change at residue 138. Of the last two changes, the valine at residue 45 (as in the published sequence) is conserved during phylogeny, whereas the glycine at residue 138 (as in pBS33) is conserved (using the alignment in reference 15). The variations in the first 6 residues (with the exception of the first glycine in pBS33, which is contributed by the linker) probably result from either alternative or incomplete splicing of the cardiac HSP90 mRNA that generated pBS33.

The cDNA from pBS33 was inserted into pmal-cR1 (New England Biolabs), a prokaryotic expression vector that generated a fusion protein composed of an N-terminal MBP fused to the C-terminal 194 amino acids of HSP90 encoded by pBS33 (the fusion protein is referred to as MBP-HSP90). The fusion protein exhibited strong reactivity with the amphipathic helix antibody (Fig. 3, lane 3) and was purified by maltose affinity column chromatography (Fig. 3, lane D). Confirmation of the molecular mass of the HSP90 fragment was accomplished by digesting the fusion protein with protease factor Xa (a specific site is encoded in the polylinker of the vector), which resulted in the generation of a band with a relative migration rate during SDS-PAGE of 23 kDa (Fig. 3, band F). This value is in agreement with the predicted molecular mass.

Treatment of MyoD1 synthesized in *E. coli* with MBP-HSP90 enhances sequence-specific DNA-binding activity. Several studies have demonstrated that MyoD1 specifically binds the consensus sequence CANNTG present in the muscle creatine kinase and other muscle-specific enhancers (24, 27). Complementary oligomers derived from the muscle creatine kinase enhancer right MyoD1-binding site (containing a single CACCTG-binding site) have previously been shown to bind MyoD1 bacterial fusion proteins in EMSAs (5). For the EMSAs presented here, the same oligomers were used with purified MyoD1 generated in a T7 polymerase-based prokaryotic expression system (purified MyoD1 was generously given by H. Weintraub, Fred Hutchinson Cancer Research Center). The reactions were performed in the absence of exogenously added ATP and in the presence of excess acetylated BSA and poly(dI-dC) (to ensure specificity). Preincubation of MyoD1 with MBP-HSP90 resulted in a substantial increase in its DNA-binding activity (Fig. 4, compare lanes 2 and 3). This effect was not observed with MBP alone (Fig. 4, lane 5), indicating that the 194-residue C-terminal fragment of HSP90 is the active region of the fusion protein. Heat-denatured soluble MBP-HSP90 also did not enhance MyoD1 DNA-binding activity (Fig. 4, lane 8), nor did the addition of equivalent amounts of recombinant HSP70 (not shown; HSP70 generously given by J. Thomas, New York University School of Medicine). MBP-HSP90 alone did not produce a mobility shift of the oligomers (Fig. 4, lane 7), and activated MyoD1 (lane 3) comigrated with control MyoD1 (lane 2), suggesting that MBP-HSP90 does not participate directly in DNA binding (further experiments, presented below, confirmed this conclusion).

Although an equivalent effect was observed with native HSP90 isolated from mouse brain (described below), we found MBP-HSP90 to be the experimental reagent of choice for several reasons: (i) native HSP90 displayed poor solubility in the buffers used for EMSA, whereas MBP-HSP90 was highly soluble; (ii) native HSP90 rapidly lost activity,

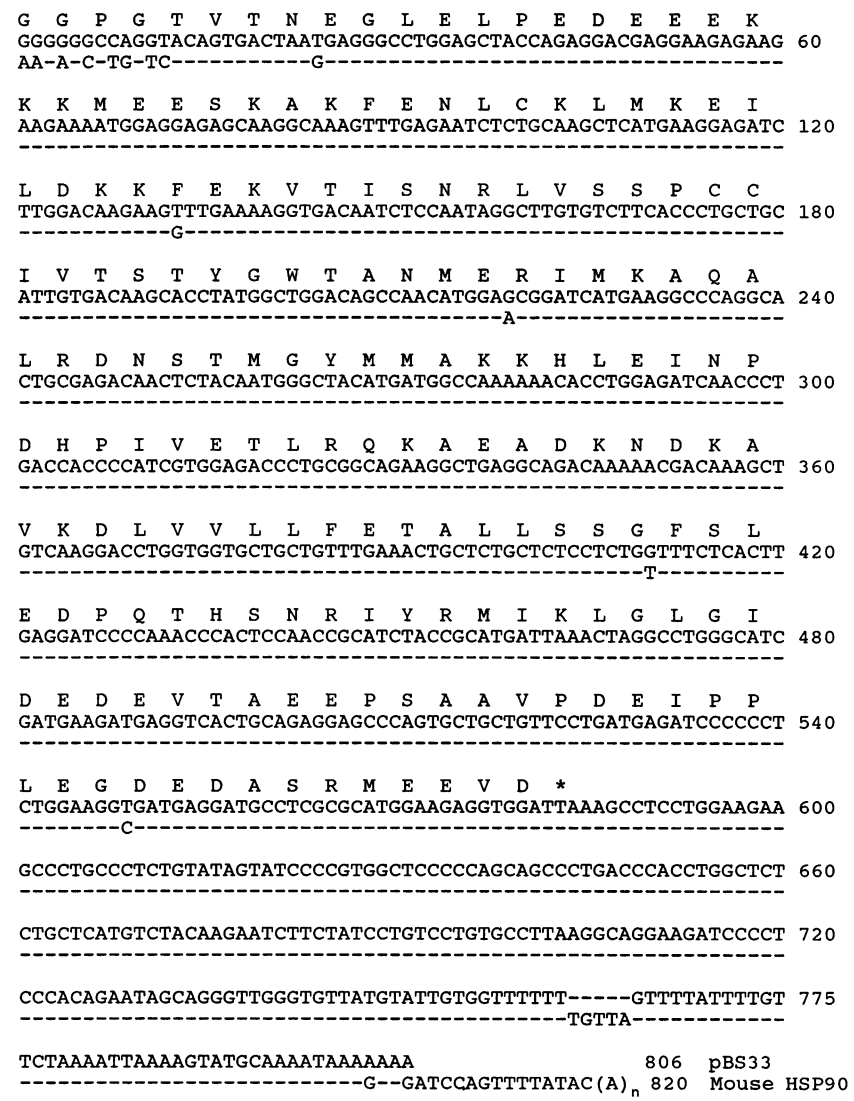

FIG. 2. Nucleotide sequence of a C-terminal fragment of murine HSP90 that was expression cloned from a λgt11 library with the amphipathic helix antibody. The nucleotide sequence of clone pBS33 is compared with the homologous region of a previously published sequence for murine HSP90 (HSP84 [26]).

whereas MBP-HSP90 was stable during storage; and (iii) MBP-HSP90, being a recombinant protein, was available in large quantities. We attempted to confirm the results presented in this report with commercially available human HSP90 (lyophilized) but found it to be completely insoluble in the EMSA buffer (even when dissolved in water first). The drawbacks associated with the use of native HSP90 are probably attributable to properties of residues 1 to 530, which are not essential for the activity described here.

Enhancement of MyoD1 DNA-binding activity results from a transient interaction between MyoD1 and MBP-HSP90. Experiments were performed to determine that MBP-HSP90 is not a permanent component of the MyoD1-DNA complex. To evaluate whether MBP-HSP90 makes contact with the DNA component of the MyoD1-DNA complex, UV cross-linking experiments were performed. MyoD1-DNA complexes were exposed to UV light for increasing lengths of time, then resolved by SDS-PAGE, and visualized by autoradiography. Cross-linked MyoD1 resolved as a monomer at 40 kDa, the predicted mass for the protein plus radiolabelled nucleotide probe (Fig. 5, lanes 1 to 7). The addition of MBP-HSP90 to the binding mixtures did not produce any novel bands (Fig. 5, lanes 8 to 14). This result indicates that MBP-HSP90 does not contact DNA present in the MyoD1-DNA complex.

Tertiary complexes are observed as more slowly migrating, supershifted bands in EMSA, and they result from the binding of other proteins (such as antibody [24]) to components of the shifted protein-DNA complexes. As shown above, the addition of MBP-HSP90 to EMSA binding mixtures did not result in the generation of a supershifted band, suggesting that MBP-HSP90 does not bind the MyoD1-DNA complex. A caveat to this, however, is that MBP-HSP90 and MyoD1 could form an active heterodimer that migrates at a rate similar to that of the homodimer. To rule out this possibility, binding reactions were performed in the presence of an MBP antibody. The presence of this antibody in the reaction mixtures also did not produce a supershifted

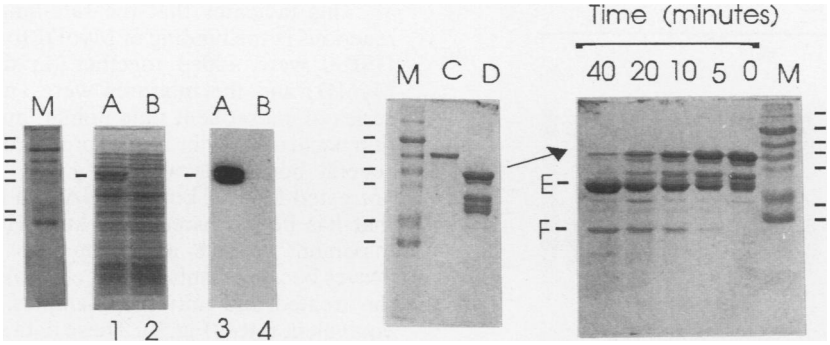

FIG. 3. Generation and purification of MBP-HSP90 fusion protein. The cDNA insert from pBS33 was shuttled to pmal-cR1 (New England Biolabs), a prokaryotic expression vehicle that expresses the *malE* gene MBP fused N terminal to a cloned insert in the same frame (for *Eco*RI) as the *lacZα* fusion products of λgt11. Lanes: M, size markers (from top to bottom, 180, 116, 84, 58, 48.5, 36.5, and 26.5 kDa); A, whole lysate of *E. coli* expressing MBP-HSP90; B, whole lysate of control *E. coli*; C, native HSP90 purified from murine brain (generous gift of S. Ullrich and E. Appella, National Cancer Institute) D, MBP-HSP90 purified by amylose affinity chromatography. For E and F, the purified product in lane D was digested with protease factor Xa (a specific cleavage site is present between the MBP and HSP90 residues). E, the MBP protein fragment; F, the 23-kDa HSP90 C-terminal fragment. Lanes: 1, 2, and lanes not numbered, Coomassie stain; 3 and 4, Western blot with amphipathic helix antibody.

band, confirming the lack of MBP-HSP90 in the MyoD1-DNA complex (Fig. 6). Together, these experiments indicate that MBP-HSP90 enhances MyoD1 DNA-binding activity but does not stably bind to either the DNA or protein components of the final complex.

Activation of MyoD1 by MBP-HSP90 occurs stoichiometrically, and the activation potential of MBP-HSP90 can be depleted. To determine whether activation of MyoD1 requires stoichiometric or catalytic amounts of either HSP90 or MBP-HSP90, dose-response experiments were performed. MyoD1 was preincubated with serial dilutions of either native HSP90 or MBP-HSP90 for 24 h (to allow completion of the reaction), then incubated with probe, and analyzed by EMSA. Native HSP90 and MBP-HSP90 displayed similar dose-response progressions, with maximum activity for both occurring between 1.8 and 3.5 molar equivalents to MyoD1 (Fig. 7). In a typical experiment, maximum activation of MyoD1 by native HSP90 resulted in a signal 2.3 times greater (Fig. 7). The actual signals resulting from treatment of 200 ng of MyoD1 with 3.5 molar equivalents of native HSP90 or MBP-HSP90 (using probes with the same specific activity) were virtually identical, and differences observed in relative activation resulted from variations in the activity of untreated MyoD1. These data show that the effect of HSP90 on MyoD1 DNA-binding activity is dose dependent and occurs as a stoichiometric interaction between the

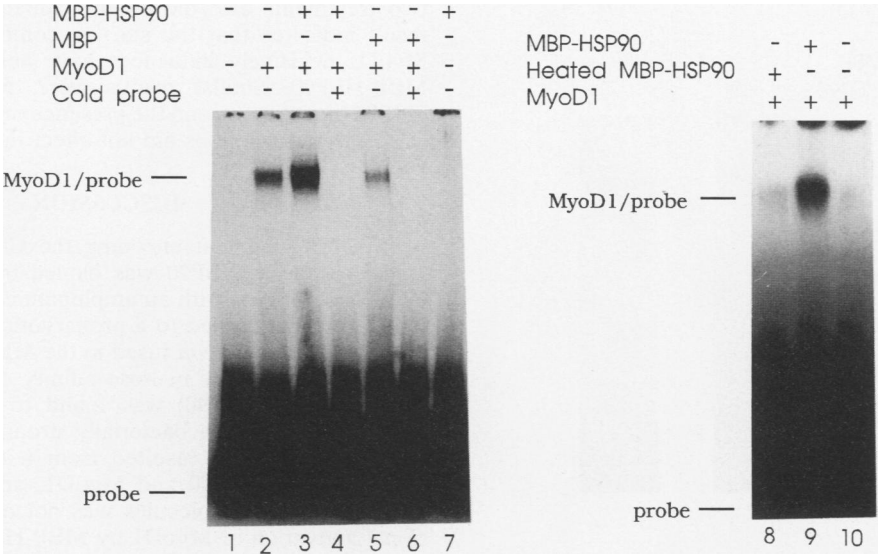

FIG. 4. Treatment of MyoD1 generated in *E. coli* with MBP-HSP90 enhances its DNA-binding activity. EMSAs were performed with MyoD1, MyoD1 treated with MBP-HSP90 (3:1 MBP-HSP90 to MyoD1 [molar ratio]), MyoD1 treated with MBP (3:1), and MyoD1 treated with heated MBP-HSP90 (70°C, 45 min; MBP-HSP90 remains soluble after this treatment). MyoD1 was pretreated with MBP-HSP90 or MBP for 2 h and then incubated with complementary oligomers (probe) derived from a MyoD1 binding site in the muscle creatine kinase enhancer (MEF1 site). MyoD1 binding activity was significantly enhanced by MBP-HSP90 treatment but slightly reduced by MBP alone. The latter result is typical and is also produced by the addition of similar quantities of immunoglobulin (see Fig. 6). Addition of heat-treated MBP-HSP90 (lane 8) did not enhance MyoD1 binding activity.

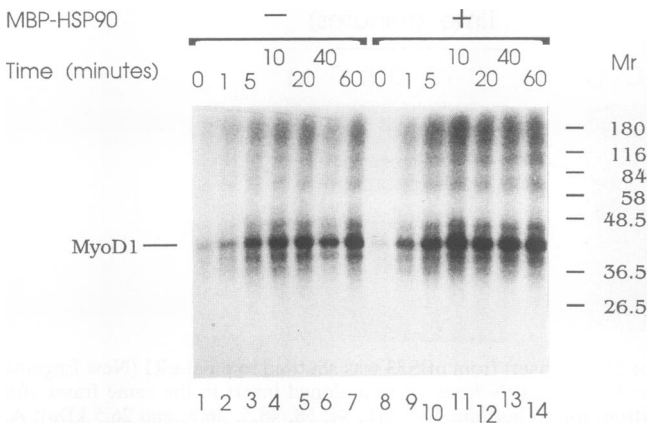

FIG. 5. Cross-linking of bound complementary oligomers to MyoD1 in the presence of MBP-HSP90 results in only MyoD1 being linked. MyoD1 (−) and MyoD1 treated with MBP-HSP90 (+) as described in the legend to Fig. 4 were treated with shortwave UV for the indicated times. The samples were then resolved by SDS-PAGE and visualized by autoradiography. A single major band was resolved at 40 kDa, which represents the mass of a single MyoD1 polypeptide bound to DNA. No qualitative difference was detected after treatment with MBP-HSP90, indicating that MBP-HSP90 does not form a heterodimer with MyoD1.

two proteins. The molar ratio of HSP90 to MyoD1 required for maximal activity suggests that HSP90 operates in the reaction as a dimer or trimer.

Time course experiments were performed to study the relative rate at which MBP-HSP90 activates MyoD1. These experiments revealed that activation of MyoD1 by HSP90 occurred more rapidly than binding to DNA. Incubation of MyoD1 with MBP-HSP90 for 10 min (4°C) prior to incubation with added probe for 1 h results in no greater activation than preincubating MyoD1 with MBP-HSP90 for 24 h (Fig. 8). This indicates that the rate-limiting step of these two reactions is the binding of MyoD1 to DNA. When probe and HSP90 were added together (in the presence of excess MyoD1) and the mixtures were analyzed by EMSA at a series of subsequent time points, maximum relative activation occurred within 1 h but probe continued to be bound for several hours afterward (Fig. 9). This result shows that untreated MyoD1 bound DNA at a lower rate than MyoD1 that has been treated with MBP-HSP90. After 1 h, when maximum relative activation was observed, the binding curves became displaced but equivalent, indicating that both the treated and untreated samples were binding probe at equivalent rates (Fig. 9). These data show that the activity of MBP-HSP90 is rapidly depleted in the presence of excess MyoD1.

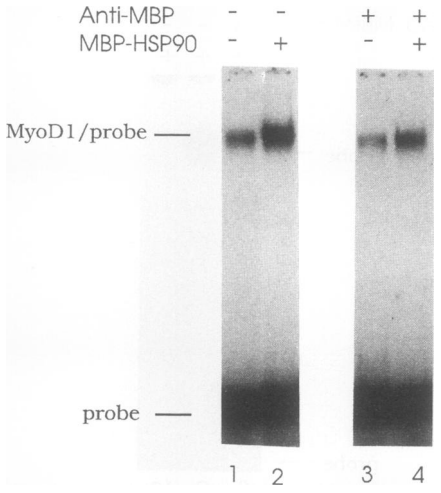

FIG. 6. MBP-HSP90 does not form a stable tertiary complex with MyoD1 and DNA. MyoD1 was treated with MBP-HSP90 in the presence (+) or absence (−) of an MBP antibody (New England Biolabs). A supershifted band was not observed in lane 4, indicating that a tertiary complex of antibody–MBP-HSP90–MyoD1 DNA was not present. This result (and that in Fig. 5) indicates that the enhancement of DNA binding by MyoD1 results from a transient interaction with MBP-HSP90.

Activation of mutant and heat-denatured MyoD1 by MBP-HSP90. Studies with deletion mutants have demonstrated that the bHLH region of MyoD1 is necessary and sufficient for DNA binding and for transdetermination to the myogenic phenotype (37). A deletion mutant of MyoD1 containing residues 57 to 167 (the cysteine- and histidine-rich [C/H] and bHLH regions) was synthesized as a glutathione-*S*-transferase fusion protein in *E. coli* and purified as described elsewhere (37) (generous gift of A. Lassar and H. Weintraub, Fred Hutchinson Cancer Research Center). The DNA-binding activity of this polypeptide was also enhanced by treatment with MBP-HSP90 (Fig. 10), although the enhancement was not as significant as that observed with full-length MyoD1. This result indicates that the recognition site for MBP-HSP90 is contained within residues 57 to 167 of MyoD1, but the mutant polypeptide after treatment with MBP-HSP90 assumes a less stable active conformation than the full-length molecule. MyoD1 is denatured with the full loss of DNA-binding activity by heating (45°C) in a low-salt buffer, repetitive freeze-thaw cycles, acetone precipitation, or incubation at low pH. Of these, treatment with MBP-HSP90 partially recovered activity from heat-denatured (Fig. 11) and low-pH-denatured (not shown) MyoD1 (these two treatments also did not precipitate the protein). This result indicates that the starting conformation of inactive MyoD1 decisively influences how well it is activated by MBP-HSP90. Similar results were obtained with native HSP90 (not shown), and the presence or absence of ATP and $MgCl_2$ in the reactions did not affect their outcome.

DISCUSSION

A cDNA fragment encoding the C-terminal 194 amino acids of murine HSP90 was cloned by screening a λgt11 expression library with an amphipathic helix antibody. The fragment was shuttled to a prokaryotic expression plasmid and produced in *E. coli* fused to the MBP of the *malE* gene. After purification by maltose affinity chromatography, the product (MBP-HSP90) was found to activate the DNA-binding activity of a bacterially produced bHLH protein, MyoD1. Activation resulted from a transient interaction between MBP-HSP90 and MyoD1, and a stable complex between the two molecules was not observed. Conformational activation of MyoD1 by MBP-HSP90 occurred more rapidly than binding to DNA, but whether MBP-HSP90 facilitated assembly of MyoD1 into dimers or activated the structure of the existing dimers has not yet been determined. Equivalent activation was observed when either native HSP90 purified from murine brain or recombinant MBP-HSP90 was used. Surprisingly, ATP was not required for the activation reaction, which occurred spontaneously at 4°C.

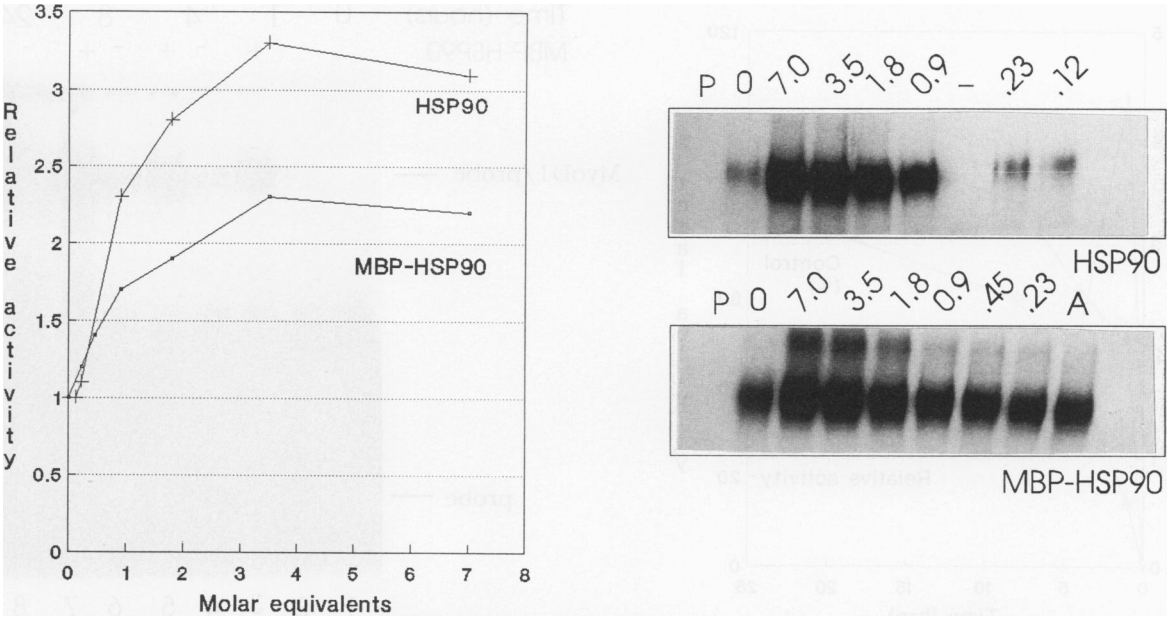

FIG. 7. Dose dependence of HSP90 and MBP-HSP90 enhancement of MyoD1 DNA-binding activity. MyoD1 was treated with a series of dilutions of native HSP90 (purified from mouse brain; kind gift of S. Ullrich and E. Appella, National Cancer Institute) and MBP-HSP90 and analyzed by EMSA. Results are quantified as relative activity, or treated band density divided by untreated band density. The amount of added MBP-HSP90 or HSP90 is indicated as molar equivalents to MyoD1 (monomer), and the maximal activity for both was observed at 3.5 molar equivalents. Preincubations were continued for 18 h (to completion) before the addition of DNA (probe). Abbreviations: P, probe alone; A, 7.0 molar equivalents of acetylated BSA added. For both experiments, identical quantities of MyoD1 and probes (with equivalent specific activities) were used, and the band intensities of the maximally activated MyoD1 produced by either MBP-HSP90 or HSP90 were equivalent. The displacement of relative activation between the two experiments is due to differing activities of the untreated MyoD1.

Since the potential of MBP-HSP90 to activate MyoD1 is depleted stoichiometrically by the reaction, its role in the reaction is that of a substrate rather than a catalyst. Other components not present in the purified protein preparations may be required in vivo to return HSP90 to its initial state and thereby make the reaction catalytic. In the present system, however, MBP-HSP90 and native HSP90 appear to consume their own conformation energy while rendering MyoD1 active for DNA binding.

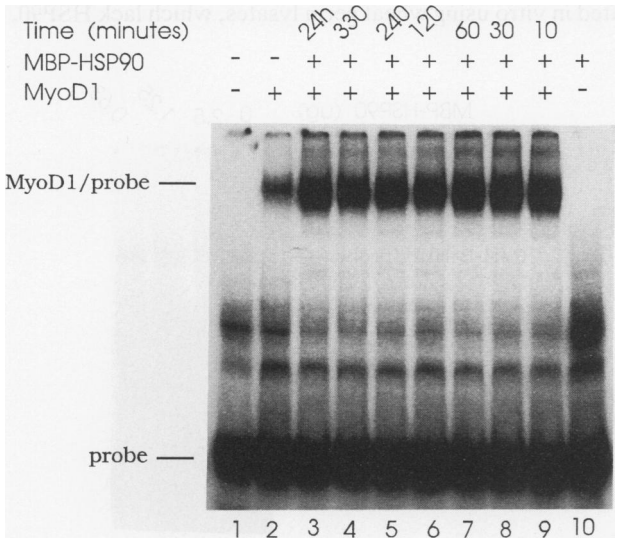

FIG. 8. Conformational activation of MyoD1 by MBP-HSP90 occurs more rapidly than binding to DNA. MyoD1 was preincubated with MBP-HSP90 for the indicated times, then incubated with probe for 2 h, and analyzed by EMSA. The time of preincubation did not significantly affect the amount of enhancement observed, and it may be concluded that DNA binding was rate limiting (see Fig. 9).

The amphipathic helix antibody found to react with the C-terminal 194 residues of HSP90 was generated by using as immunogens synthetic polypeptides representing the second amphipathic helices of the HLH regions of Myc and MyoD1 (Fig. 1). Preincubation of the antibody with an excess of the MyoD1 peptide completely abolished binding to MBP-HSP90, suggesting that the structural homolog on HSP90 more closely resembles the second amphipathic helix of MyoD1 than that of Myc. Since the *myc* peptide partially inhibited binding of the antibody to MyoD1 but not to MBP-HSP90, the second helix of Myc may bear more homology to that of MyoD1 than to the antibody recognition site on MBP-HSP90. Although the actual epitope(s) on MBP-HSP90 with which the antibody reacts has not been identified experimentally, a large amphipathic helix is predicted between residues 28 and 58 (558 to 588 on full-length murine HSP90) by using an α-helix algorithm described by Chou and Fasman (8) and hydropathy parameters described by Kyte and Doolittle (20). As indicated in Fig. 12, the predicted amphipathic helix is conserved and contains repeated hydrophobic residues consistent with a leucine zipper (LZ) structure (23). Recent evidence has suggested that the LZ of at least one protein (c-Jun [2]) physically associates with the HLH of MyoD1. The putative LZ identified in Fig. 12 may mediate a similar (but transient) association between MyoD1 and HSP90. Since dose-response experiments presented in this report suggest that HSP90 operates as a dimer, this LZ may also be responsible for the generation of a coiled-coil structure between two HSP90 monomers. Exper-

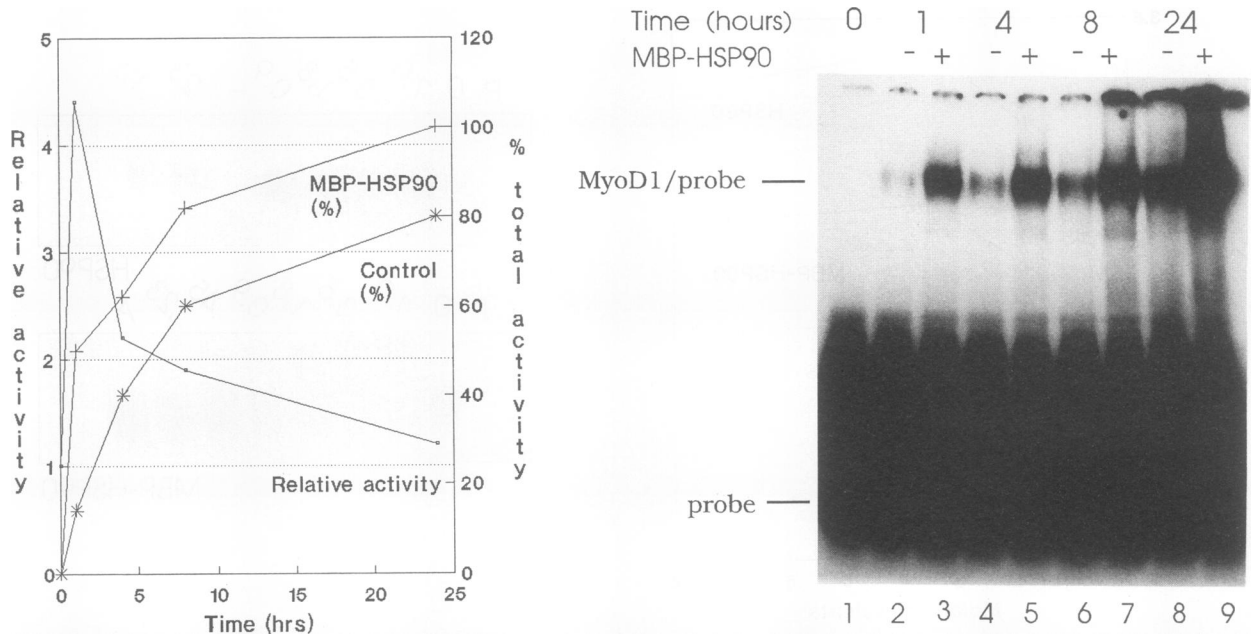

FIG. 9. The activation potential of MBP-HSP90 can be depleted. MyoD1, in the presence (+) or absence (−) of 1 molar equivalent of MBP-HSP90, was incubated with probe for the indicated times and analyzed by EMSA. Maximal relative activation (density of the treated MyoD1 band divided by density of the untreated MyoD1 band) was observed at 1 h. At 4, 8, and 24 h the increases in total activation (arbitrarily shown as percentages of lane 9 density) of treated and untreated MyoD1 were parallel, indicating that the activation potential of MBP-HSP90 was depleted. This is also shown as a decrease in relative activity.

iments with mutant HSP90 polypeptides should yield more insights into the functional significance of this motif and its role in conformational activation of MyoD1.

The most significant effect of HSP90 treatment in this study was observed when pristine bacterial MyoD1 was used, followed at substantially lower levels by heat- and pH-denatured (not shown) MyoD1. This suggests that HSP90, which is expressed at high levels under normal physiological conditions, may function primarily in folding newly synthesized polypeptides rather than those denatured by stress. This conclusion is supported by other studies, particularly those conducted with yeast mutants synthesizing 20-fold-lower levels of their HSP90 homolog (30). Glucocorticoid receptor synthesized in these mutants produced significantly less hormone-induced transcriptional activation than receptor synthesized in normal strains. In addition, other studies have shown that glucocorticoid receptor translated in vitro using wheat germ lysates, which lack HSP90, is

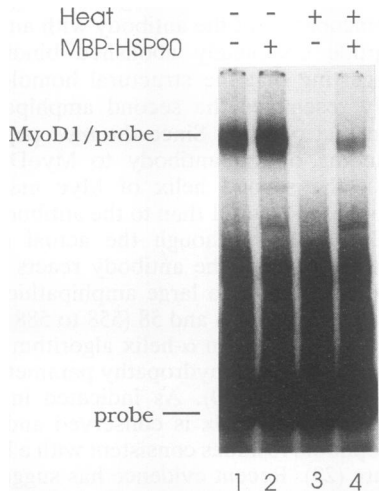

FIG. 10. The deletion mutant of MyoD1 containing only the C/H-bHLH regions is activated by MBP-HSP90. Glutathione-*S*-transferase fusion protein (200 ng) containing the cysteine- and histidine-rich (C/H) and bHLH domains of MyoD1 (see Materials and Methods; generous gift of A. Lassar and H. Weintraub, Fred Hutchinson Cancer Research Center) was treated with MBP-HSP90 and analyzed by EMSA.

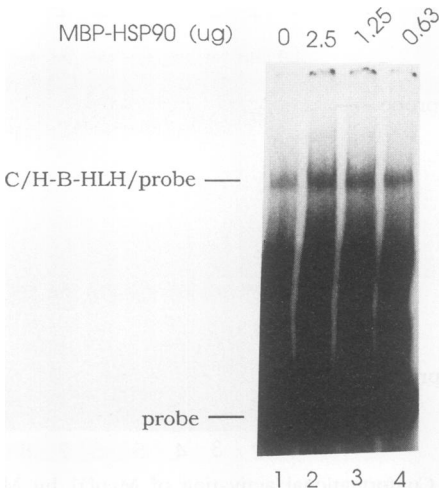

FIG. 11. The DNA-binding activity of heat-denatured MyoD1 is partially restored by MBP-HSP90. MyoD1 and MyoD1 denatured by heating to 45°C in low-salt buffer were treated with MBP-HSP90 (3.5 molar equivalents; 18-h preincubation) and analyzed by EMSA. DNA-binding activity was partially restored.

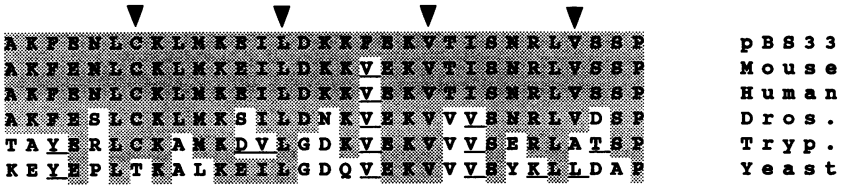

FIG. 12. The LZ structure is conserved in HSP90. Protein sequence alignment of clone pBS33 (residues 28 to 58) with mouse (residues 558 to 588 of HSP84 [26]), human (HSP89β [32]), *Drosophila* (Dros. [3]), *Trypanosoma cruzi* (Tryp. [10]), and *Saccharomyces cerevisiae* (Yeast [11]) HSP90s. The area indicated is predicted to be a large amphipathic helix, and the positions indicated by arrowheads contain conserved hydrophobic residues arranged in a LZ motif. Grey area shows identical residues; underlines indicate conserved substitutions.

less active than receptor translated in reticulocyte lysates, which contain HSP90. In this report, MyoD1 synthesized in *E. coli* is shown to be more active after treatment with a fragment of murine HSP90. Unlike the glucocorticoid receptor and the AH receptor (which binds HSP90 but has not yet been shown to be conformationally activated by it), however, MyoD1 does not form a stable complex with HSP90 (shown by immunoprecipitation experiments in reference 37). This observation suggests that a number of other proteins may be conformationally activated by HSP90 either during or after their synthesis but have eluded detection because they do not form a stable complex with it. The abundance of HSP90 supports the notion that it interacts with a number of proteins other than those with which it forms a stable complex and that those forming a stable complex probably represent a specialized minority.

As is indicated by the presence of its homologs in organisms as diverse as yeasts and humans, HSP90 has been functionally important through the course of animal phylogeny. Consequently, more recent appearances (such as MyoD1) have evolved their functions in the presence of HSP90, and it is not surprising that such proteins could be conformationally dependent on it for activity. Future experiments should be aimed at better understanding which classes of proteins are dependent on HSP90 and whether any structural motifs are particularly well suited for conformational activation by it. It may not be coincidental that many of the targets of HSP90 identified so far are transcription factors, and additional experiments should explain its fundamental role in their conformational biochemistry.

ACKNOWLEDGMENTS

We thank Steven Ullrich and Ettore Appella (National Cancer Institute) for the gift of purified murine brain HSP90 (HSP84), Harold Weintraub (Fred Hutchinson Cancer Research Center, Seattle, Wash.) for the T7 MyoD1, Andrew Lassar and Harold Weintraub for the Glu-MyoD1 deletion mutant, and John Thomas (New York University School of Medicine) for the gift of recombinant HSP70.

This work was supported by grants HL40659 and HL43583 from the National Institutes of Health.

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