Hsp90 Phosphorylation Is Linked to Its Chaperoning Function

ASSEMBLY OF THE REOVIRUS CELL ATTACHMENT PROTEIN*

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Studies on Hsp90 have mainly focused on its involvement in the activation of several families of protein kinases and of steroid hormone receptors. Little is known regarding the role of Hsp90 in the folding of nascent proteins. We previously reported that Hsp90 plays an active role in the posttranslational assembly of the C-terminal globular head of the reovirus attachment protein σ1. We show here that Hsp90 becomes phosphorylated in this process. However, only the unphosphorylated form of Hsp90 is complexed with σ1, suggesting that Hsp90 phosphorylation is coupled to the release of the chaperone from the target protein. Geldanamycin, which blocks σ1 maturation by preventing the release of Hsp90 from σ1, also inhibits Hsp90 phosphorylation. Taken together, these results demonstrate that Hsp90 phosphorylation is linked to its chaperoning function.

Cellular chaperones are a group of proteins whose major roles appear to be the prevention of target protein aggregation and the promotion of their correct folding and assembly (1–3). Chaperones such as Hsp70 and Hsp40 likely interact with a wide variety of polypeptides, and thus their involvement in the folding processes is believed to be of a general and universal nature. Another major molecular chaperone, Hsp90, appears to be more specific in terms of “client” selection and has been shown to interact mainly with proteins involved in transcription regulation and signal transduction pathways, such as steroid hormone receptors and protein kinases (4–8). Apparently Hsp90 stabilizes these proteins and keeps them in a conformation amenable to activation under appropriate conditions. Although the extent of Hsp90 involvement in the general folding scheme of cytosolic proteins remains unclear, it has been recently suggested, from studies on Drosophila, that Hsp90 plays a major role in morphological evolution (9). It was proposed that Hsp90 normally suppresses morphogenic variations that become manifest upon Hsp90 impairment, leading to abrupt evolutionary changes.

Although it is generally accepted that Hsp90 coordinates with other chaperones to promote the folding and assembly of the target protein by a binding and release mechanism, precisely how these processes are regulated remains unclear. Recent evidence suggests that assembly of the glucocorticoid receptor-Hsp90 complex involves the sequential involvement of Hsp70 and Hsp90, both being ATP-dependent events (10). That ATP binding and hydrolysis are essential for Hsp90 function was also demonstrated using the progesterone receptor (11). ATP binding apparently also results in conformational changes in Hsp90 concomitant with its association with the cochaperone p23 (4). Another less well studied phenomenon relates to Hsp90 phosphorylation, which may represent yet another level of regulation of Hsp90 function. Hsp90 can be phosphorylated in vitro at two serine residues by casein kinase II (12) and at two threonine residues by DNA-dependent protein kinase (13). In addition, Hsp90 was shown to enhance the kinase activity of eukaryotic initiation factor 2a kinase but only after prior phosphorylation of Hsp90 by casein kinase II (14). In another study, treatment of cells with the serine/threonine phosphatase inhibitor okadaic acid led to hyperphosphorylation of Hsp90 and decreased association between Hsp90 and pp60-src, suggesting a link between Hsp90 phosphorylation and target protein interaction (15).

The role of Hsp90 in the folding of newly synthesized protein has not been extensively probed. Hartson et al. (16) studied the folding of the lymphoid cell kinase p56lck translated in vitro and demonstrated the association of Hsp90 with newly synthesized p56lck molecules. It was further revealed that although the Src homology 2 domain folds independently of Hsp90, folding of the catalytic domain (C-terminal to the Src homology 2 domain) is Hsp90-dependent. Subsequent phosphorylation at the C-terminal domain of p56lck correlated with stabilization of the kinase, which is no longer associated with Hsp90. Another study using the heme-regulated eukaryotic initiation factor 2a kinase translated in vitro shows that Hsp90 plays an obligatory role in this kinase acquiring and maintaining a conformation that is competent for transformation into an aggregation-resistant activable kinase (17).

The in vitro translation system has also been used extensively in our laboratory to reveal the mechanisms of folding and assembly of the reovirus cell attachment protein σ1, a trimeric protein positioned at the 12 vertices of the icosahedral virion (18–22). The σ1 trimer is highly asymmetric, with an N-terminal fibrous tail that is anchored to the virion and a C-terminal globular head that interacts with the cell receptor (23–26). Evidence from in vitro translation studies has revealed that these two structurally distinct domains are generated by separate trimerization events (18). The N-terminal fibrous tail is highly α-helical and contains an extended heptad repeat of hydrophobic residues, ending this region with the intrinsic

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propensity to form a triple coiled-coil. During α1 biogenesis, assembly of three neighboring nascent chains occurs cotranslationally (i.e. on the polysome) at the N terminus (19, 20). This process does not involve any chaperones or ATP and results in the generation of a loose triple coiled-coil. As the trilex moves down the polysome, chaperones (e.g. Hsp70) begin to interact with the emerging residues of the elongating C termini, preventing their misfolding and aggregation. Protein α1 leaves the polysome as a partially assembled trimer with some chaperones attached to the unassembled C termini. This type of α1 is known as the “unstable hydra form” because it migrates as monomers even under “nondissociating” conditions (without boiling) in SDS-PAGE (18, 19). Subsequent ATP-dependent release of chaperones presumably provides the opportunity for the loose coiled-coil to quickly snap together (tightening the coiled-coil), whereas the remaining portions of the three C termini are available for continued interaction with chaperones. This structure, with a stably assembled N terminus and an unassembled C terminus, is called “stable hydra form,” and it migrates as a retarded trimer in SDS-PAGE under nondissociating conditions. Further ATP-dependent release and re-binding of chaperones leads to global assembly and folding of the C terminus, generating mature α1 with the characteristic lollipop-shaped structure (called the “mature compact form”), which migrates as an unretarded trimer in SDS-PAGE under nondissociating conditions. We have proposed that the involvement of two mechanistically distinct oligomerization events for the same molecule, one cotranslational and one posttranslational, may represent a common approach to the generation of oligomeric proteins in the cytosol (19).

Our previous demonstration that Hsp90 is involved in the posttranslational assembly of the α1 globular head (21) has led us to question the possible role of Hsp90 phosphorylation in this process. In the present study, we demonstrate that Hsp90 phosphorylation occurs during the posttranslational maturation of α1 and is coupled to the assembly of the globular head. However, only the unphosphorylated form and not the phosphorylated form of Hsp90 is complexed with immature α1, suggesting that Hsp90 phosphorylation is linked to the release of the chaperone from the target protein. Inhibiting this release using the benzoxquinone ansamycin, geldanamycin (GA), also abrogates Hsp90 phosphorylation. These observations have led us to propose that Hsp90 phosphorylation is linked to its chaperoning function.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and in Vitro Translation—The plasmids encoding the full-length and various truncated α1 products have been described previously (18, 27). All transcripts were generated in vitro using the MEGAscript™ Transcription Kit (Ambion) for the Sp6 polymerase promoter. A typical transcription reaction involved incubation of 1 μg of linearized plasmid DNA with the prescribed contents of the Ambion Express Kit (total final volume of 20 μl for 5 min at 37 °C). The mRNA product was isolated by LiCl precipitation followed by cleanup with the Bio101, Inc. RNaid Kit. The purified mRNA was then resuspended in 0.1% diethyl pyrocarbonate-treated water to a final concentration of 0.5 μg/μl and stored at −70 °C for future use.

Transcripts were translated in vitro in rabbit reticulocyte lysate (Promega) according to the manufacturer’s specifications. Typically, for analysis of translation products, 0.5–1.0 μg of mRNA was incubated at 37 °C with 7 μCi of [35S]methionine (Amersham Pharmacia Biotech), 1 μl of 1.0 mM methionine minus amino acids (Promega), and 20 μl of rabbit reticulocyte lysate (Promega) for the duration described in the figure legends.

In Vitro Phosphorylation Assay—Typically, translation was carried out for 37–9 min at 37 °C in prewarmed rabbit reticulocyte lysate. These lysates may or may not have been supplemented with [35S]methionine, depending upon the experiment (see the figure legends). Two microliters of [γ-32P]ATP was then added to each reaction (final radio-specific activity, 2 μCi/μl), and incubation was continued for the indicated times. For posttranslational chase experiments, translation was halted by either centrifugation (to pellet ribosomes) or addition of cycloheximide (final concentration, 30 μg/ml). [γ-32P]ATP was then added, and the reactions were incubated further at 37 °C for the periods described in the figure legends. The reactions were routinely supplemented with the 20 μM leupeptin (Sigma) as protease inhibitor. Geldanamycin was prepared as a stock solution of 175 μg/ml in 20% Me2SO, which was added to the reaction mixture to a final concentration of 7 μM at the onset of the chase (unless stated otherwise in figure legends).

In Vitro Phosphorylation of Hsp90 Proteins—Purified bovine Hsp90 was obtained from Stressgen Inc. Rabbit Hsp90 was partially purified from rabbit reticulocyte lysate according to the protocol of Ianniotti et al. (28). Labeling of bovine Hsp90 was carried out by incubating 5 μg of protein in 35 μl of kinase buffer (20 mM Tris-Cl, pH 7.2, 20 mM KCl, 0.5 mM MgCl2, 60 mM NaCl, 10 mM sodium metabisulfite, 20 mM β-mercaptoethanol, 10 μM ATP, 20 μM [γ-32P]ATP, and 20 μM NaCl) containing 7 μCi of [γ-32P]ATP and 1 unit of casein kinase II for 15 min at 37 °C. The partially purified preparation of rabbit Hsp90 protein was similarly labeled but in the absence of added casein kinase II.

SDS-PAGE—Discontinuous SDS-PAGE was performed using the procedures of Laemmli (29). All reactions were analyzed by staining the SDS-PAGE gel with Coomassie blue or autoradiography or were silver stained as described in this paper.

RESULTS

Translation of Full-length α1 Protein Triggers p86 Phosphorylation—We have previously shown that Hsp90 interacts with the C-terminal half of protein α1 and that it is required for α1 maturation (i.e. assembly of the C-terminal globular head) (21). Because Hsp90 is a known phosphoprotein, we decided to investigate the phosphorylation status of Hsp90 during α1 assembly. To this end, full-length α1 was translated (for 15 min) in rabbit reticulocyte lysate in the presence of [γ-32P]ATP, and these reactions were immunoprecipitated with a monoclonal anti-Hsp90 antibody, 12G3. At the time point chosen for immunoprecipitation, our previous studies have shown that the majority of the α1 protein has trimerized within the N-terminal coiled-coil domain and a subpopulation has progressed to the mature form that has also folded within the C-terminal globular domain (21). Analysis of the immune pellet by SDS-PAGE revealed that a protein migrating at the ~86-kDa position was phosphorylated (Fig. 1A, left lane). In contrast, immunoprecipitates

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; GA, geldanamycin; FL, full-length.
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from a translation reaction of a truncated σ1 (mutant d294) representing the N-terminal one-third of the protein, which does not associate with Hsp90 (21), did not contain a [32P]-labeled 86-kDa protein (Fig. 1A, right lane). This demonstrates that phosphorylation of the 86-kDa protein (p86) is σ1 C-terminus-dependent and not a by-product of translation events. Comparison of the migration rate of phospho-p86 with that of rabbit phospho-Hsp90 and bovine phospho-Hsp90 (both precipitable with 3G3) reveals that the three proteins have identical apparent molecular masses (Fig. 1B). These experiments therefore suggest that p86 is likely Hsp90 and is referred to as Hsp90 henceforth.

Phosphorylation of Hsp90 Occurs during Posttranslational Processing of σ1—We previously showed that assembly of the C-terminal globular head of σ1 occurs posttranslationally (19) and that this process involves Hsp90 (21). It would therefore be of interest to see whether phosphorylation of Hsp90 is also a posttranslational event. To this end, σ1 mRNA was translated only long enough to generate immature trimers. These molecules have been previously characterized as the earliest posttranslational folding intermediate of σ1 and possess loosely wound trimeric N termini and unfolded C termini (previously designated the unstable hydra form) (20, 21). At least three chaperones are associated with these structures: Hsp90, p23, and Hsp70 (21). The reactions were then centrifuged at high speed to pellet polysomes, thereby preventing further translation. The supernatants, now containing only posttranslational maturation of σ1, were translated in rabbit reticulocyte lysate for 8 min at 37 °C. The samples were then transferred to ice and then centrifuged to pellet the ribosomes and halt translation. Supernatants were then removed, and [γ-32P]ATP was added to them. They were then incubated at 37 °C for the indicated times, immunoprecipitated (IP) with the 3G3 anti-Hsp90 antibody, and analyzed by SDS-PAGE.

Phosphorylation of Hsp90 occurs during posttranslational maturation of σ1. FL σ1 and d294 transcripts were separately translated in rabbit reticulocyte lysate for 8 min at 37 °C. The samples were then transferred to ice and then centrifuged to pellet the ribosomes and halt translation. Supernatants were then removed, and [γ-32P]ATP was added to them. They were then incubated at 37 °C for the indicated times, immunoprecipitated (IP) with the 3G3 anti-Hsp90 antibody, and analyzed by SDS-PAGE.

The results (Fig. 2) show that phosphorylation of Hsp90 occurred to a significant degree during further folding of the full-length σ1 but was not detectable for the C-terminal truncation mutant d294 (control), although comparable amounts of the two polypeptides were synthesized in these reactions (not shown). Similar results were obtained when cyclohexamide was used to halt translation reactions (data not shown). These results therefore suggest that phosphorylation of Hsp90 is a posttranslational event and is linked to σ1 C-terminal assembly. However, they provide no information as to whether Hsp90 phosphorylation occurs only when the target protein is folded correctly or whether interaction of Hsp90 with target sites alone is enough to trigger phosphorylation, even in the absence of maturation folding of the target protein.

Hsp90 Phosphorylation Is Induced Independent of Folding but Is Dependent upon Hsp90/Target Protein Interaction—If binding to the target protein alone was sufficient to trigger Hsp90 phosphorylation, then σ1 mutants that contained a binding domain for Hsp90 but that could not properly fold should also trigger this phosphorylation. On the other hand, if phosphorylation was folding-dependent, then these mutants should be incapable of inducing Hsp90 phosphorylation. To resolve this issue, we used both N-terminal and C-terminal deletion mutants of σ1. The N-terminal deletion mutants included d1 and d1II (see schematic in Fig. 3A), both of which lack the coiled-coil trimerization domain and therefore do not trimerize (18). Extensive studies have shown that N-terminal trimerization is a prerequisite for assembly of the C-terminal globular head (18, 19). However, because both mutants contain the C-terminal half of σ1, they are nonetheless capable of interacting with Hsp90 (21). For C-terminal deletion mutants, we examined d30, d90 (lacking the C-terminal 30 and 90 residues, respectively; however, both still contain Hsp90 binding sites (21)), and d294, which contains no Hsp90-binding sites as negative control. All three C-terminal deletion mutants can trimerize within their N-terminal coiled-coil domains but fail to fold within vestigial regions C-terminal to it.

We first tested for Hsp90 association with these two families of truncation mutants by immunoprecipitation of [35S]methionine- and [γ-32P]ATP-labeled reaction mixtures with the 3G3 antibody. (The reactions were also immunoprecipitated with an anti-σ1 antibody to ensure that all σ1 constructs were expressed at comparable levels; Fig. 3B, left panel.) Immunoprecipitation with the 3G3 antibody (Fig. 3B, right panel) revealed that all nascent proteins except d294 were associated with Hsp90. We then examined the ability of each product to trigger phosphorylation of Hsp90 in these same reactions by analyzing [32P]-labeled Hsp90 precipitable with 3G3. The results (Fig. 3C) show that with the exception of d294, all the deletion mutants were able to trigger Hsp90 phosphorylation to at least the same degree as FL σ1. Thus the ability of these mutants to induce phosphorylation directly correlates with their ability to bind Hsp90, demonstrating that phosphorylation of Hsp90 is dependent on Hsp90/target protein interaction alone and not on the maturation (proper folding) of the target protein.

Phospho-Hsp90 Is Not Associated with the Target Protein—It was of interest to determine whether the phosphorylated form of Hsp90 was in association with or free from the target pro-
and suggesting no association between these two proteins. On the fluorography for the detection of 35S-labeled, and then analyzed by SDS-PAGE, followed by antibody (Fig. 4A), except the reaction was further incubated at 37 °C for 7 min. Aliquots of the reaction mixture were then immunoprecipitated (IP) with either an anti-α1 antibody directed against the N terminus of α1 (designated anti-N) or the 3G3 antibody to Hsp90, and analyzed by SDS-PAGE and autoradiography. B, same as A, except the reaction was precipitated with another anti-Hsp90 antibody (designated H90-10) and compared with immunoprecipitation with 3G3. C, same as A, except the reaction was first precleared (precleared) with the H90-10 antibody and then immunoprecipitated with the 3G3 antibody, the H90-10 antibody (Ab), and normal rabbit serum (NRS, as control).

**DISCUSSION**

Although it has been known for some time that Hsp90 is a phosphoprotein and that it can be phosphorylated under certain *in vitro* conditions, the significance of this phosphorylation event in Hsp90 function is entirely unknown. In the present study, we investigate the possibility that Hsp90 phosphorylation is linked to its chaperoning function, specifically in the folding and assembly of nascent polypeptides. We show that Hsp90 is phosphorylated during the *in vitro* biogenesis of the reovirus attachment protein α1 in rabbit reticulocyte lysate. This phosphorylation is not detectable when the N-terminal portion of α1 is translated and is strictly dependent on the presence of the C-terminal portion of the protein. This is consistent with our previous demonstration that the N-terminal fibrous tail of α1 trimerizes in a chaperone/ATP-independent manner, whereas formation of the C-terminal globular head requires both chaperones (including Hsp90) and ATP. Interestingly, the phosphorylated form of Hsp90 is not associated with α1, suggesting that Hsp90 phosphorylation is linked to release of the chaperone from the target protein. Support for this notion has come from the demonstration that GA, which inhibits the release of Hsp90 from α1, also blocks Hsp90 phosphorylation. These observations have led us to suggest that Hsp90 phosphorylation is part of the Hsp90 cycling mechanism and likely plays an important role in its chaperoning function.

A most interesting observation from our present study is that the C terminus of α1, when translated as a truncated protein and therefore unable to undergo assembly (i.e. trim-
Fig. 5. GA inhibits Hsp90 phosphorylation. A, FL s1 transcripts were translated in vitro in the presence of [35S]methionine for 10 min. The reaction was then centrifuged to pellet the ribosomes, and the supernatant was incubated further at 37 °C for 8 min. Aliquots were then added to the reaction mixtures that were further incubated for 8 min. Aliquots of the mixtures were then immunoprecipitated (IP) with the 3G3 anti-Hsp90 antibody and analyzed by SDS-PAGE.

Fig. 6. Model for Hsp90 phosphorylation/dephosphorylation in target protein folding. Only unphosphorylated or hypophosphorylated Hsp90 (complexed with p23) can interact with the unfolded or partially folded target protein. Subsequent dissociation of the Hsp90-target protein complex is coupled to Hsp90 phosphorylation and enhanced maturation of the target protein, which likely also involve other chaperones such as Hsp70, Hsp, and Hsp40 (not shown). Reiterative cycles of binding and release concomitant with Hsp90 dephosphorylation and phosphorylation lead to the generation of the mature (properly folded) target protein.
lease of Hsp90 from the substrate being associated with Hsp90 phosphorylation.

Overall, the data presented in this study are in agreement with a model wherein Hsp90 phosphorylation is intimately linked to its chaperoning function. It also appears that this phosphorylation is not necessarily accompanied by correct folding of the substrate because binding and release of Hsp90 occurs normally for mutant targets and perhaps even more efficiently than for the wild type target because the end point (generation of a properly folded final product) can never be achieved. It remains to be seen whether Hsp90 phosphorylation is coupled to the ATP binding and ATPase activity of Hsp90. However, considering the highly coordinated nature of chaperone-mediated protein folding, this is most likely the case. It also seems safe to assume that other chaperones are also actively involved in this process. Clearly other systems (e.g. steroid hormone receptors and protein kinases) will need to be probed; the demonstration that Hsp90 phosphorylation also occurs in these systems would provide further support for the notion that phosphorylation/dephosphorylation represents a key regulatory mechanism for chaperone function.

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