Histidine 89 is an essential residue for Hsp70 in the phosphate transfer reaction

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Abstract Autophosphorylation of Hsp70 is detected in the process of substrate refolding in the presence of adenosine triphosphate (ATP) in the reaction mixture. But to date, the role and mechanism of Hsp70 autophosphorylation have not been elucidated. In this study we determined the site of histidine phosphorylation of Hsp70 as an intermediate in the process of phosphate transfer reaction by site-directed mutagenesis. We selected two possible sites (ie, His89 and His227) of intermediate histidine phosphorylation based on our hypothesis of the transfer of γ -phosphoryl groups and replacement by glycine and serine. Although an acid labile autophosphorylation intermediate of Hsp70 and its cytidine diphosphate-dependent dephosphorylation were detected in wild-type Hsp70, they were markedly suppressed in the H89S mutation of Hsp70, but not on the H227S mutation. The ATPase activity and ATP synthesis activity of Hsp70 were almost completely suppressed in the H89S and H89G mutations. The role of His89 in the phosphate transfer reaction of Hsp70 is discussed.

INTRODUCTION

The 70-kDa heat shock protein (Hsp70) family constitutes an evolutionarily conserved group of molecular chaperones that are involved in diverse roles in eukaryotic cells, including folding and intracellular targeting of newly synthesized proteins, as well as regulation of signal transduction proteins and transcription factors (Bukau and Horwich 1998; Becker and Craig 1994). The molecular chaperone activity and conformational switch are functionally linked with adenosine triphosphate (ATP) hydrolysis and its interactions with protein substrates are regulated by ATP hydrolysis and adenosine diphosphate–ATP exchange (Hiromura et al 1998).

Two metal-binding sites were recently identified in the crystal structure of hATPase. The first calcium binding site appears to be important for ATP hydrolysis and in vitro phosphorylation (Sriram et al 1997). The second calcium is tightly coordinated on the human Hsp70 ATPase domain (hATPase) protein surface by Glu231, Asp232, and a carbonyl of His227. This metal-binding motif is in close proximity to the first catalytic site, and participates

both in ATP hydrolysis and ATP synthetic activities in cooperation with the first metal-binding site (Fig 1) (Sriram et al 1997).

Phosphothreonine was postulated as an intermediate of ATP hydrolysis; in the presence of divalent metal ions the activated γ -phosphate can be transferred to Thr204, and this structurally conserved residue was suggested as a phosphate acceptor (Leustek et al 1991; McCarty and Walker 1991; Gaut and Hendershot 1993). However, point mutations of Thr199 (equivalent to Thr204 in DnaK) significantly lowered, but did not completely abolish ATPase activity, and mutational studies suggest that phosphothreonine may not be an obligatory intermediate (O'Brien and McKay 1993), so an alternative side chain can serve as a phosphate acceptor. The His89 is sited between and is structurally related to the calcium-binding domains based on our X-ray crystallography studies, and we propose that His89 may serve as a phosphate acceptor between these two functional domains.

To test our hypothesis, we carried out biochemical and functional characterization research on wild-type Hsp70 and its mutant. We constructed and characterized a new Hsp70 derivative in which His89 is replaced by serine and glycine in order to examine what effect a conservative substitution of these two residues has on Hsp70 func-

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Fig 1. Interactive proximity between the first and second metal-binding sites of Hsp70. Crystal structure of the human Hsp70 ATPase domain (Protein Data Bank code for Hsp70 ATPase domain, 1hjo[30]). Two metal-binding sites were recently identified in the crystal structure of hATPase. The first binding site appears to be important for ATP hydrolysis and in vitro phosphorylation (Sriram et al 1997). The second is tightly coordinated on the hATPase protein surface by Glu231, Asp232, and carbonyl of His227. His-89 is in close proximity to these two metal-binding sites.

tion. Consequently, we found that an acid labile autophosphorylated intermediate of Hsp70 and its cytidine diphosphate (CDP)-dependent dephosphorylation were formed in wild-type Hsp70. They were markedly suppressed in the H89S and H89G mutants but not in the H227S mutant. ATP synthesis and ATP hydrolysis activities were both markedly suppressed in H89S and H89G mutants. Our results indicated that Hsp70 catalyzes intramolecular or intermolecular ATP synthesis by transferring γ -phosphate, because His89 is an essential residue in formation of the autophosphorylated intermediate.

MATERIALS AND METHODS

Materials

Nucleoside diphosphate (NDP) kinase (EC 2.7.4.6) from bovine liver, [8- 14 C]ATP, [8- 14 C]ADP and [γ - 32 P]ATP were obtained from NEN Life Science Products. All other reagents were commercially available products of the highest grade.

Construction of mutant plasmids

The mutagenic oligonucleotide was 5'-GTGGTGCAGTCG GACATGAAGAGTTGGCCTTTCCAGGTA-3' for H89S, 5'-GTGCAGTCGGACATGAAGGGCTGGCCTTTC-3' for H89G, and 5'-GGCCGGGGACACCAGCCTGGGTGGGG AGGA-3' for H227S (the mismatched bases are underlined). Mutagenesis occurred with the reagents and protocols of the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The presence of the correct mutation was confirmed in all constructs by DNA sequencing.

Expression and purification of mutant proteins

Mutant and wild-type recombinant human Hsp70 proteins were overexpressed in *Escherichia coli* BL21 (DE3) cells and it was necessary to grow the cells at 30°C to obtain fully active protein in our assays. The cultures were grown in Luria-Bertani (LB) broth and induced at an optical density of 0.8 at 600 nm by addition of isopropyl-1-thio- β -D-galactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation and lysed immediately or stored frozen at -80°C.

Lysis occurred by sonication in 10 mM imidazole, 20 mM phosphate sodium, and 0.5 M NaCl, pH 7.4. Lysate was cleared by centrifugation for 30 minutes at 12 000 $\times$ g. The cleared lysate was further purified to homogeneity by a HisTrap kit for purification of His-tagged proteins (Amersham Pharmacia Biotech). High-performance liquid chromatography was performed on a gel permeation TSK-Gel DEAE-5PW column. Briefly, recombinant protein in Buffer A (25 mM Tris-HCl pH 7.2, 0.1 mM ethylenediamine tetraacetic acid [EDTA], and 0.5 mM dithiothreitol) was applied to the column and then eluted with a narrow salt gradient of 0–0.15 M KCl in Buffer A, which is able to separate conventional NDP kinase from Hsp70 (Leung and Hightower 1997). Hsp70 was dialyzed against approximately 0.1 M KCl and judged to be >99% pure by the absence of any visible bands other than 70 kDa on a Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The concentration of protein samples was determined using Bio-Rad protein assay solution following a standard protocol.

Detection of autophosphorylated Hsp70 intermediate

Autophosphorylation and CDP-dependent dephosphorylation of Hsp70 were analyzed using 10 μ g of wild-type Hsp70 and mutant Hsp70, 10 μ Ci of [γ - 32 P]ATP (6000 Ci/mmol), 100 μ M ATP, and 6 mM MgCl₂ in 100 mM Hepes-KOH buffer pH 8.0, in the absence and presence of 5 mM CDP, respectively, in a total volume of 10 μ L. After incubation at 37°C for 2 hours, both reactions were

quenched by the further addition of 10 mM EDTA, half of each sample was treated with the traditional (pH 6.8) SDS sample buffer without boiling and subjected to 15% SDS-PAGE. After electrophoresis, the gel was dried without acid fixation and analyzed with an imaging analyzer. The remaining samples were analyzed for Hsp70 and base stability of the phosphorylated Hsp70 as described (Hiromura et al 1998). These samples were then analyzed with an imaging analyzer.

Phosphoamino acid analysis

The autophosphorylated proteins were subjected to alkaline hydrolysis in 3 M KOH for 100 minutes at 120°C. After hydrolysis, potassium ions were precipitated by slow addition of 10% perchloric acid until a neutral pH was approached, and the ions were then removed by centrifugation (Frejje et al 1997). The supernatant was spotted onto a 20 × 20-cm silica gel thin-layer chromatography (TLC) plate (J. T. Baker, Inc) along with phosphoamino acid standards and developed in *t*-butyl alcohol/methyl ethyl ketone/acetone/methanol/H₂O/NH₄OH (10:20:20:5:40:5, v/v). Phosphoamino acid standards were visualized by spraying the plate with 0.25% ninhydrin in *n*-butyl alcohol (Frejje et al 1997). Phosphoamino acid analysis of the acid-stable phosphorylated proteasome was also performed (Hunter and Sefton 1980). The hydrolysates were then analyzed by TLC and autoradiography.

Assaying ATP hydrolysis and ATP synthesis

Although almost all the data for Hsp70 ATPase reported were obtained by measurement of inorganic phosphate liberated from ATP, we found that Hsp70 exhibits the reverse activity (ie, adenosine diphosphate [ADP]-ATP exchange), and thus the amount of inorganic phosphate in the reaction is not reflected by the ATPase activity. In this regard, we analyzed the ATPase activity by measuring the conversion of [¹⁴C]ATP to [¹⁴C]ADP. The ATP synthesis activity was analyzed by measuring the conversion of [¹⁴C]ADP to [¹⁴C]ATP as described (Braell et al 1984; Flynn et al 1989).

The reactions were carried out at 37°C for 30 minutes in 100 mM Hepes-KOH buffer pH 8.0 containing 5 mM ATP, 0.5 mM ADP, and 6 mM MgCl₂ with 0–20 pmol of Hsp70 (monomeric form), and 0.05 μCi of [8-¹⁴C]ATP and 0.02 μCi of [8-¹⁴C]ADP to assay ATPase and ATP synthesis activities, respectively, in a total volume of 10 μL. After incubation, the reaction mixtures were immediately spotted onto a polyethyleneimine-cellulose TLC plate (Macherey-Nagel). ADP and ATP were separated by ascending chromatography with 1 M formic acid containing 0.7 M LiCl, and the radioactivity in the resolved spots

was quantitated with a Bio Imaging Analyzer BAS 1500 (Fuji Photo Film Co Ltd, Japan).

RESULTS

Expression and purification of recombinant Hsp70 proteins

Wild-type human Hsp70 complementary DNA was cloned into a pET-21a(+) vector and site-directed mutants of Hsp70 were made according to the method described earlier.

Cells carrying the Hsp70 overexpressing plasmid pET-2a were grown at 37°C to late log phase (*A*₆₀₀ ~600) in 1 L of LB medium with ampicillin. Recombinant wild-type and mutated Hsp70 proteins produced by IPTG-induced BL21 (DE₃) *E. coli* transformed cells in a 1 mM final concentration and cultured for another 2 hours at 30°C. The cells were pelleted by centrifugation (3000 × *g*, 30 minutes, 4°C). Further protein purification and measurements were performed as described in Materials and Methods. Hsp70 was judged to be >99% pure by the absence of any visible bands other than Hsp70 on a Coomassie brilliant blue-stained SDS-PAGE gel.

Formation of an autophosphorylated Hsp70 intermediate and its CDP-dependent dephosphorylation

Hsp70 gave a single autophosphorylated intermediate band corresponding to a protein with a molecular mass of 70 kDa in the absence of the nucleoside diphosphate, CDP, in the reaction mixture, which contained 10 μCi of [³²P]ATP, 100 μM ATP, and 6 mM MgCl₂ (Fig 2, lane 1). In the presence of 5 mM CDP in the reaction mixture, however, the radioactivity of the phosphorylated intermediate decreased significantly (Fig 2, lane 2). Furthermore, a significant decrease in the radioactivity of the autophosphorylated intermediate was also observed on the H89S mutant group with and without 5 mM CDP added to the reaction mixture. These results indicate that Hsp70 catalyzes the transfer of the γ -phosphate group from ATP to CDP, and that this transfer involves the formation of a phosphoenzyme intermediate. To characterize the phosphorylation of Hsp70, autophosphorylated Hsp70 was subjected to acid, neutral, and basic treatments as described (Leustek et al 1989; Yano et al 1999) to evaluate a high-energy phosphate on a histidine residue of protein. The phosphorylated intermediate of Hsp70 was stable upon alkaline treatment, but labile upon acid treatment (20% trichloroacetic acid, with a significant decrease in the autophosphorylation level as compared with neutral treatment, as shown in Fig 2). The result suggests that the acid-labile phosphorylated His89 residue may be responsible for the formation of phosphorylated intermediate in wild-type Hsp70.

Fig 2. SDS-PAGE of Hsp70, autophosphorylation of Hsp70 in a neutral and acid labile manner, and CDP-dependent dephosphorylation. The autophosphorylation and CDP-dependent dephosphorylation of Hsp70 (10 μ g) were analyzed as described in Materials and Methods. The autophosphorylation of wild-type Hsp70 and the H89S mutant of Hsp70 in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 5 mM CDP for 2 hours. Half of each sample was treated with the traditional (pH 6.8) SDS sample buffer without boiling, subjected to 15% SDS-PAGE, and then dried without acid fixation. For acid stability, the remaining halves of phosphorylated Hsp70 were boiled in the SDS sample buffer at pH 6.8 and then electrophoresed. The gel was fixed in 20% trichloroacetic acid, followed by Coomassie brilliant blue staining, destaining in methanol/acetic acid, and drying (lanes 5–8). The samples were then analyzed with an imaging analyzer.

Phosphoamino acid analysis

To further study the character of the phosphorylated intermediate in our experiment, we performed phosphoamino acid analysis, and the different experiment groups were examined. The negative group was treated without addition of Hsp70. NDP kinase was applied in the positive group. We also set up a control group of Thr204 mutant in our study. The results are shown in Figure 3; phosphohistidine was detected.

The results show that phosphohistidine is formed in the NDP kinase, wild-type, and Thr204 mutant groups, but phosphohistidine was not formed in the His89 mutant or in the group with hydrochloric acid (ie, HCl treated). These results further indicate that the acid labile His89 residue is an essential residue for formation of a phosphorylated intermediate in biochemical functional processes of Hsp70. Hsp70 catalyzes the transfer of the γ -phosphate group from ATP to the His89 residue to form a phosphorylated intermediate. We found that compared with the H89S mutant, the H227S mutant maintained, for the most part, autophosphorylation levels.

Identification of ATPase activity and ATP synthesis activity of wild-type and mutant Hsp70

Because Hsp70 proteins exhibit ATP hydrolysis as well as ATP synthesis, the reverse activity, the amount of inorganic phosphate in the reaction mixture is not directly reflected by ATP hydrolysis activity in the presence of ADP in the reaction mixture. We thus analyzed ATP hydrolysis activity by measuring the conversion of [14 C]ATP to [14 C]ADP in the presence of 0.5 mM ADP, and the ATP synthesis activity was analyzed by measuring the conversion of [14 C]ADP to [14 C]ATP.

We found the recombinant Hsp70 that was free from NDP kinase exhibited ATPase activity. As shown in Figure 4A, Hsp70 exhibited weak ATPase activity in a dose-dependent manner, but lower activity was observed for the H89 mutant protein.

The reverse reactions of ATP hydrolysis, ATP synthesis (Fig 4B), of wild-type Hsp70 and mutants of Hsp70, were

also analyzed. The results show that wild-type Hsp70 catalyzed ATP synthesis at a slow but linear rate in the presence of substrate ADP in a dose-dependent manner. Compared with the wild-type Hsp70, we found that ATPase activity and ATP synthesis activity of H89 mutants were suppressed more than those of the wild-type protein.

DISCUSSION

It has been proposed that the release of a target protein by Hsp70 is coupled to ATP hydrolysis through a conformational change, which is induced by an autophosphorylation event. Members of the Hsp70 family have also been shown to be phosphorylated *in vivo* (Loomis et al 1982; Welch et al 1983), and the level of phosphorylation is known to be influenced by stress and other factors (Rieul et al 1987; Hendershot et al 1988). Finally, DnaK and the eukaryotic Hsp70 homologs localized in mitochondria (P71), chloroplasts, and the endoplasmic reticulum (BiP/Grp78) have recently been shown to undergo a Ca^{2+} -stimulated autophosphorylation reaction (Cegielska and Georgopoulos 1989; Amir-Shapira et al 1990).

The crystal structure of the human ATPase domain shows two metal-binding sites: the first, a catalytic center, is essential for ATP binding; and the second site, in co-operation with the first metal-binding site, is for ADP binding in the reactions of ATP hydrolysis and ATP synthesis (Sriram et al 1997). His89 was postulated to be a phosphate acceptor intermediate between these two functional domains in our study; the autophosphorylation level in the H89S and H89G mutants was significantly more suppressed than it was in the wild-type Hsp70 protein. Autophosphorylated Hsp70 intermediate and its CDP-dependent dephosphorylation activity were also found in wild-type Hsp70. With further characteristic study of the phosphorylated amino, we demonstrated that His89 is essential for formation of the autophosphorylated intermediate in Hsp70. Hsp70 shows NDP kinase-like activity instead of ATPase activity, which catalyzes the transfer of γ -phosphoryl groups from nucleoside triphosphates to

Fig 3. Phosphoamino acid analysis of phosphorylated wild-type and mutants of Hsp70. (A) The phosphoamino acid analyses of the autophosphorylated NDP kinase from human erythrocytes (16 μg) as a positive standard (lane 6), negative control (lane 1), wild-type Hsp70 (lane 2), H89S mutant (lane 3), Thr204 mutant (lane 4), and the wild type of Hsp70 treated with 6 N HCl (WT + HCl, lane 5) were performed as described in Materials and Methods. Phosphohistidine was synthesized from polyhistidine as described (Wei and Matthews 1991). Positions of ^{32}P -phosphohistidine ($^{32}\text{P-His}$) and phosphate (Pi) are indicated. (B) Phosphoamino acid analyses of H227S (lane 4), H89S (lane 3), and wild-type Hsp70 (lane 2). The control group (lane 1) was set up without addition of protein.

Fig 4. ATP hydrolysis and ATP synthesis activities of Hsp70 and its mutant. (A) ATP hydrolysis activities of Hsp70 and H89S were assayed as described in Materials and Methods using 0–60 pmol of each protein. Five millimolar ATP, 0.05 μCi [^{14}C]ATP, 6 mM MgCl_2 , and 0.5 mM ADP. After a 1-hour reaction at pH 8.0, samples were analyzed by thin-layer chromatography, and ADP formation was calculated with an imaging analyzer. (B) The ATP synthesis activities of wild-type Hsp70 and its H89S mutant were assayed as described in Materials and Methods using 0.02 μCi [^{14}C]ADP. The ATP synthesis activities of Hsp70 were also examined at 37°C for 1 hour in the reaction mixture containing 0.5 mM ADP.

NDPs through autophosphorylated histidine as an intermediate. Therefore, based on the results, we believe His89 is the most likely candidate residue in formation of the phosphorylated intermediate of Hsp70 in that Hsp70 catalyzes the transfer γ -phosphate from ATP to His89 to

form the phosphorylated intermediate first, then these γ -phosphates are further transferred to CDP or to other residues.

The autophosphorylation level in the control group of His227 remained unchanged. His227 is related to the second calcium-binding site in X-crystal structure; further studies on this domain may be necessary in the future, and would be interesting.

ATPase activity and ATP synthesis of the H89 mutant were suppressed primarily in the wild-type group in a dose-dependent manner. The exchange reaction exhibited a preference for ATP as a phosphate donor and specificity for CDP as a phosphate acceptor, and yielded autophosphorylated intermediates during the transfer of γ -phosphate from ATP to ADP. From these results, it is likely that autophosphorylation of Hsp70 is directly coupled to ATP hydrolysis and synthesis activity, and that His89 plays an essential role in these processes.

Two calcium sites were identified in the crystal structure of hATPase. The first calcium binds within the catalytic pocket, bridging ADP and inorganic phosphate. In this active domain, the conserved Lys71 is identified as a catalytically essential residue that affects ATP hydrolysis (O'Brien et al 1996). The second calcium site represents a new calcium-binding motif that can play a role in the stabilization of the protein structure (Sriram et al 1997). These two active domains form a functional channel that is related to ATP-ADP exchange activity in Hsp70.

From our experiment, His89 acts as an important residue in transferring the γ -phosphate in this functional channel. It can form a phosphorylated intermediate in the functional process of Hsp70, and a mutation in His89 will affect the essential ATP-ADP exchange activity in Hsp70. As we know, in the Hsp70-protein/peptide interaction mechanism, ADP-ATP exchange is rate-limiting in the cycle of substrate binding and release, and the intrinsic ADP-ATP exchange activity of Hsp70 may affect the ATP-dependent reaction cycle of protein folding. The conformational change was induced by a change in the ATPase and ATP binding activity in its ATPase domain; the further effect on the chaperone function of Hsp70 was produced with the conformational change on the protein structure, so the ATP-ADP exchange activity in ATPase domain was identified as being essential to the chaperone function of Hsp70.

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