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The C-terminal Helices of Heat Shock Protein 70 Are Essential for J-domain Binding and ATPase Activation*[S]

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Xue-Chao Gao[‡], Chen-Jie Zhou[‡], Zi-Ren Zhou[‡], Meng Wu[‡], Chun-Yang Cao[§], and Hong-Yu Hu[‡1]

From the [‡]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China and the [§]State Key Laboratory of Bio-organic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 200032, China

Background: HSJ1a can bind with HSP70 to regulate many cellular events.
Results: The C-terminal helices of HSP70 contribute to its interaction with HSJ1a J-domain and stimulation of ATPase activity.
Conclusion: The C-terminal helical subdomain is crucial for modulating J-domain interaction and allosteric activation.
Significance: This finding provides an alternative mechanism of allosteric activation for functional regulation of HSP70 by its J-domain co-chaperones.

The J-domain co-chaperones work together with the heat shock protein 70 (HSP70) chaperone to regulate many cellular events, but the mechanism underlying the J-domain-mediated HSP70 function remains elusive. We studied the interaction between human-inducible HSP70 and *Homo sapiens* J-domain protein (HSJ1a), a J domain and UIM motif-containing co-chaperone. The J domain of HSJ1a shares a conserved structure with other J domains from both eukaryotic and prokaryotic species, and it mediates the interaction with and the ATPase cycle of HSP70. Our *in vitro* study corroborates that the N terminus of HSP70 including the ATPase domain and the substrate-binding β-subdomain is not sufficient to bind with the J domain of HSJ1a. The C-terminal helical α-subdomain of HSP70, which was considered to function as a lid of the substrate-binding domain, is crucial for binding with the J domain of HSJ1a and stimulating the ATPase activity of HSP70. These fluctuating helices are likely to contribute to a proper conformation of HSP70 for J-domain binding other than directly bind with the J domain. Our findings provide an alternative mechanism of allosteric activation for functional regulation of HSP70 by its J-domain co-chaperones.

The 70-kDa heat shock proteins (HSP70)[2] are the most ubiquitous and important chaperones that function in many biological events including protein folding and assembly, post-translational modification, transportation, and secretion, and degradation (1). The HSP70 protein is comprised of an N-terminal 44-kDa ATPase domain (also called nucleotide-binding domain, NBD) and a C-terminal substrate-binding domain (SBD), which can be further divided into an 18-kDa β-sandwich subdomain (also called SBD-β) forming a hydrophobic binding pocket, and a C-terminal 10-kDa α-helical subdomain (SBD-α) that acts as a lid over the binding pocket (2, 3). The multiple functions of HSP70 are well regulated in cells by different J-domain (JD) co-chaperones, such as HSP40 (4). The J domain-containing co-chaperones are required for regulating the ATPase activity of HSP70 and subsequently modulate the substrate binding and ATPase cycle (5).

Homo sapiens J domain protein (HSJ1), also called DNAJB2, is a J domain-containing co-chaperone and preferentially expressed in neural tissues (6). There are two alternatively spliced isoforms identified, HSJ1a and HSJ1b. HSJ1a has been reported to function in regulating the ATPase activity of HSP70 and substrate binding (7, 8). Our current research has revealed that HSJ1a can regulate the proteasomal degradation of polyglutamine protein ataxin-3 by dual association with HSP70 and ubiquitin (8). Although it is well known that the J domain of HSJ1a (HSJ1a_JD) is responsible for HSP70 binding and subsequent substrate fate determination, the mechanism of the interaction between HSJ1a_JD and HSP70 and the regulation has not been understood yet.

Since the 1990s, several structures of J domains from different protein species have been solved and their interactions with HSP70 have also been well studied (9–11). It is well established that the J domain, particularly its conserved HPD motif, specifically binds to HSP70 (12). However, details of the J-domain binding to mammalian HSP70 has not been clearly addressed, and the molecular mechanism of the J-domain binding and ATPase activation is still obscure. Previous study showed that the ATPase domain of DnaK, a prokaryotic homolog of mammalian HSP70, provides the basic binding mode for the J domain of DnaJ (13). The crystal structure of auxilin J domain complexed with a C-terminally truncated form of bovine HSC70 was also solved (14), but the two molecules were covalently linked together by a disulfide. This structure of HSC70/auxilin_JD complex revealed that both the nucleotides and J domain modulate the linker conformation and the NBD-SBD interactions of HSC70.

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The atomic coordinates and structure factors (codes 2LGW and 2LMG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[S] This article contains supplemental Table S1 and Figs. S1–S8.

[1] To whom correspondence should be addressed. E-mail: hyhu@sibs.ac.cn.

[2] The abbreviations used are: HSP, heat shock protein; HSJ, *H. sapiens* J-domain protein; NBD, nucleotide-binding domain; JD, J-domain; Trx, thioredoxin; SBD, substrate-binding domain.

Our current study indicates that HSJ1a dually regulates the protein levels of ataxin-3 through binding of the J domain with HSP70 and the UIM domain with polyUb chains (8). Based on biochemical investigation, we herein report that the C-terminal helical subdomain of HSP70 is essential for binding with the J domain of HSJ1a, and the specific binding is the basis for stimulating the ATPase activity of HSP70. The binding of the J domain to HSP70 may cause an allosteric activation of the ATPase domain of HSP70 through conformational fluctuation of the C-terminal subdomain and thus the entire HSP70 protein.

EXPERIMENTAL PROCEDURES

Construction of the Expression Plasmids—The encoding sequences for HSJ1a (residues 1–274) and HSJ1a_JD (1–91, 1–75) were amplified via PCR. The PCR products were cloned into pGEX-4T-3 or pET22b$^+$ vector for bacterial expression using BamH I/XhoI or NdeI/XhoI cloning sites. HSP70 (residues 1–641) and its fragments (1–384, 1–561, 1–595, 1–610, and 1–631) were cloned into pET22b$^+$ expression vector (15). HSP537–610 was cloned to pET32M vector to express thioredoxin (Trx)-fused protein. All constructs were confirmed by DNA sequencing.

Protein Expression and Purification—The plasmids encoding His-tagged or GST fusion proteins were expressed in *E. coli* BL21 (DE3) cells. The His-tagged proteins were purified through a Ni^{2+}-NTA column (Qiagen) according to manufacturer's instructions. GST and its fusion proteins were purified using the glutathione Sepharose-4B column (Amersham Biosciences). The HSP537–610 peptide was obtained by cleaving the Trx-fused protein with thrombin. The ^{15}N- and $^{15}N/^{13}C$-labeled HSJ1a_JD and HSP537–610 proteins were prepared by using the M9 minimal medium containing ^{15}N-NH_4Cl and/or $^{13}C_6$-D-glucose, and purified by a Ni^{2+}-NTA affinity column. All the proteins were further purified by Superdex-75 column chromatography on FPLC (GE Healthcare).

Circular Dichroism (CD)—Circular dichroic spectra were obtained by using a J-715 spectropolarimeter (JASCO) as previously described (16). Experiments were performed at room temperature in 2.5 mM Tris-HCl (pH 8.0) buffer at a protein concentration of 0.2 mg/ml. All CD spectra were recorded in the wavelength range of 190–250 nm and processed by averaging three scans of a sample.

GST Pull-down Experiment—The purified GST or GST fusion proteins were incubated with glutathione Sepharose-4B beads in a PBS buffer (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.3) at 4 °C for 1 h. The His-tagged proteins were incubated with the immobilized GST or GST-fusion proteins at 4 °C. After incubating for 2 h, the beads were collected by centrifugation and washed four times with the same buffer, and then eluted by a GSH buffer (50 mM Tris-HCl, 10 mM GSH, pH 8.0). The samples were then subjected to SDS-PAGE, followed by Coomassie blue staining.

NMR Spectroscopy—$^{15}N/^{13}C$-labeled HSJ1a_JD (1–91) or HSP537–610 with a concentration of ~1 mM was dissolved in a buffer containing 20 mM phosphate, 50 mM NaCl, pH 6.5. All of the heteronuclear spectra were recorded at 25 °C on a 600-MHz NMR spectrometer. Sequence-specific assignments for the J domain were performed as described (17). For NMR titration experiments, ^{15}N-labeled J-domain protein at the molar concentration of 0.2 mM was used to be titrated with HSP70 and its C-terminally truncated fragments, and then the [^1H, ^{15}N] HSQC spectra were acquired to monitor the peak intensity changes upon titration (18).

Fluorescence Analysis—Fluorescence measurements were performed on a Varian Cary Eclipse spectrofluorometer (Agilent Technologies). The intrinsic fluorescence of HSP70 proteins (~20 μM) was measured in a 500-μl buffer (50 mM Tris-HCl, 20 mM NaCl, pH 7.5) when aliquots (5 μl) of acrylamide (stock 5 M) were added. The excitation wavelength was set at 295 nm and the emission was recorded in the range of 315–380 nm. The data were plotted as fluorescence intensity ratio (F_0/F) against acrylamide concentration and analyzed by using the modified Stern-Volmer equation, $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are the fluorescence intensities in the absence or presence of quencher, [Q] is the concentration of quencher, and K_{SV} is the Stern-Volmer quenching constant (19, 20).

ATPase Activity Assay—Malachite green based colorimetric assay (21, 22) was utilized to measure inorganic phosphate released by HSP70 and its fragments for the ATPase activities. The reaction was set in 2-ml assay buffer (50 mM Tris-HCl, pH7.0; 20 mM NaCl, 2 mM $MgCl_2$) at 37 °C. A mixture of 1 μM ATPase, 2 μM HSJ1a_JD and 500 μM ATP was incubated in a constant temperature system and each 200 μl of the reaction sample was taken out from the mixture at different time points (0, 5, 10, 15, 20, 25, 30 min) and 50 μl of stop buffer (500 mM EDTA, pH8.0) was added immediately. Then 750 μl of malachite green reagent (0.1% malachite green, 1% ammonium molybdate, 1 M HCl, filtered through a Φ0.45-μm membrane) was added to each sample and allowed stay for 5 min. The absorbance of the samples was read on a spectrophotometer at 650 nm. Sodium dihydrogen orthophosphate monohydrate was used as phosphate standard. The enzymatic activity was represented as the initial rate for hydrolyzing ATP to phosphate (A_{650}/min).

Segmental Isotopic Labeling—To uniformly label the segment (537–610) with ^{15}N in the context of HSP1–610, we used the IMPACT-CN system according to the manufacturer's guidance (NEB) (23). Briefly, HSP1–536 was cloned to the pTYB1 expression vector with NdeI/SapI sites to get the protein fragment with a C-terminal α-thioester. The N-terminal Ser residue of the fragment HSP537–610 was mutated to Cys for combination. Then the C-terminally labeled HSP1–610 was obtained by ligation of the C-terminal thioester of HSP1–536 and the ^{15}N-labeled HSP537–610. The TROSY-based [^1H,^{15}N] HSQC spectra were recorded in an 800-MHz NMR spectrometer (Bruker Biospin).

RESULTS

The J Domain of HSJ1a Regulates the ATPase Activity of HSP70—The previous studies showed that the HSP70 proteins have a very weak basal ATPase activity, and the J-domain proteins function to stimulate their ATPase activities (24, 25). To understand the mechanism underlying ATPase activity stimulation of human inducible HSP70 by HSJ1a, we divided the whole HSP70 protein into several C-terminally truncated frag-

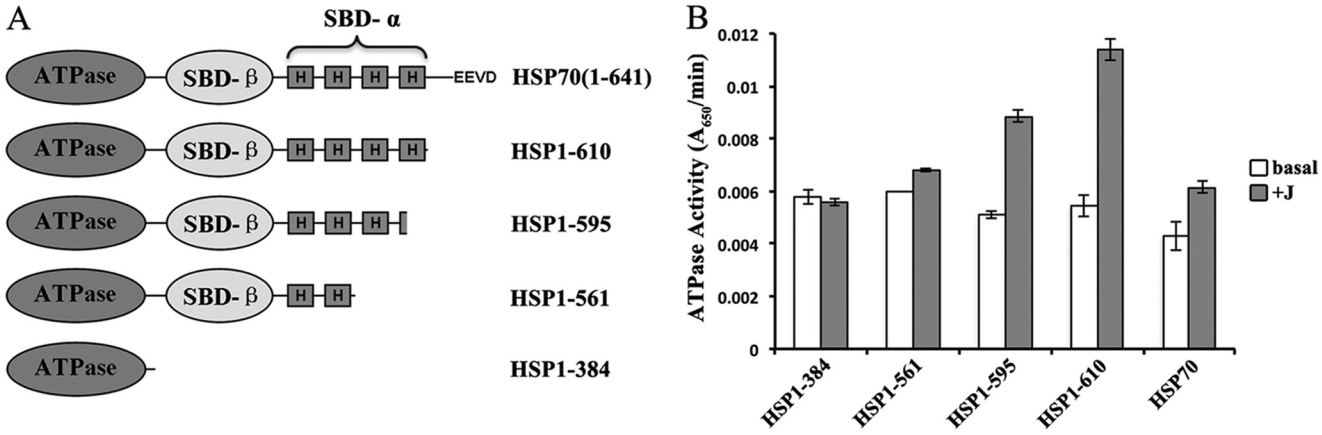

FIGURE 1. **The J domain of HSJ1a regulates the ATPase activities of various HSP70 forms.** *A*, domain architecture of HSP70 highlighting the C-terminal helices. ATPase, the N-terminal 44-kDa ATPase domain; SBD-β, the substrate-binding domain; SBD-α, the C-terminal four-helix subdomain. *B*, HSJ1a_JD stimulates the ATPase activities of HSP70 and its C-terminally truncated fragments. The ATP hydrolysis activities of various HSP70 forms (1 μM) were measured with or without addition of HSJ1a_JD (2 μM). These HSP70 forms include full-length HSP70 (1–641), HSP1–610, HSP1–595, HSP1–561, and HSP1–384 (ATPase domain). Shown are means ± S.D. ($n = 3$).

ments based on the domain architecture (Fig. 1*A*). The ATPase activities of purified HSP70 were measured by malachite green based colorimetric assay (21). The initial rates of various HSP70 fragments are significantly stimulated to different extents by addition of the J domain (residues 1–91) of HSJ1a (HSJ1a_JD) (Fig. 1*B*), though the basal ATPase activity of each fragment is similarly low. The N-terminal ATPase domain of HSP70 (HSP1–384), which binds and catalyzes the hydrolysis of ATP, does not show a stimulation of the ATPase activity in the presence of the J domain. This is consistent with the previous result from DnaK/DnaJ pair that the J domain of DnaJ failed to stimulate ATP hydrolysis of DnaK1–385, as revealed by single turnover ATPase activity measurements (27). The fragment that encompasses both the ATPase domain and the substrate-binding domain (HSP1–561) displays a slight increase of the ATPase activity in the presence of HSJ1a_JD. Compared with the full-length HSP70, the fragment HSP1–595 that has its half of the last C-terminal helix deleted also shows a significant activation of the ATPase activity. However, only the fragment HSP1–610 that retains its intact C-terminal helical subdomain exhibits great stimulation of the ATPase activity by the J domain, even this activation is more efficient than that of full-length HSP70.

To verify whether truncation of the C-terminal helices causes significant conformational change of HSP70, far-UV CD spectra of the fragments were recorded and compared (supplemental Fig. S1). These truncation mutants share similar secondary structures, suggesting that the low activation of the helix-deleted mutants by HSJ1a-JD is unlikely to be caused by loss of the overall structure.

As for the J domain of HSJ1a, we also examined the stimulation function of the JD fragment with residues 1 - 75. This fragment can also considerably stimulate the ATPase activities of HSP1–610 and full-length HSP70 (supplemental Fig. S2), indicating that the glycine/phenylalanine (G/F)-rich region (72–91) does not affect the function of the J domain. Even in the case of 10-fold excess of the J domain, the ATPase activities of HSP1–384 and HSP1–561 still cannot be effectively stimulated (supplemental Fig. S2*A*). These results demonstrate that the C-terminal helices of HSP70 (residues 562–610) play crucial roles in the ATPase activation by the J domain of HSJ1a.

Solution Structure of the J Domain of HSJ1a—As reported, the J domain of HSP40 (DnaJ) can interact with the ATPase domain of HSP70 (DnaK) (13), while the G/F-rich region flanked by the J domain of DnaJ plays a role in the DnaK chaperone cycle (28). To get mechanistic insights into the interaction between HSJ1a and HSP70, we solved the solution structure of the J domain of HSJ1a by NMR techniques. We prepared a construct that encoding both the J domain and the G/F-rich region of HSJ1a (HSJ1a_JD, residues 1–91). The G/F-rich region is highly flexible in solution, so we just calculated the structure of the J domain (1–71) (supplemental Table S1). The structure of HSJ1a_JD is mainly comprised of four α-helices with residues 4–7, 16–29, 40–56, and 59–68, respectively (Fig. 2, *A* and *B*). A loop of residues 30–39 is characterized by a highly conserved ^{31}HPD motif. This structure is very similar to those of other conserved J domains (10, 11). In comparison of these structures, HSJ1a_JD is more similar with HSP40_JD (Fig. 2*C*) than DnaJ_JD (Fig. 2*D*), giving the RMSDs of 4.5 Å and 5.5 Å, respectively, suggesting that interaction of HSJ1a_JD with HSP70 might adopt a similar mode with HSP40_JD (11). Thus, the domain structure analysis indicates that HSJ1a_JD is capable of interacting with HSP70 similar to HSP40_JD.

The ATPase and Substrate-binding Domains of HSP70 Are Not Sufficient for HSJ1a J-domain Binding—Although the function of HSJ1a and its interaction with HSP70 has been studied since the identification of HSJ1a (7), we still do not know whether the C-terminal helical subdomain contributes to the specific interaction and the ATPase activation. To demonstrate the binding mode of HSP70 with HSJ1a_JD, we firstly studied physical interaction between full-length HSP70 or its C-terminally truncated fragments and HSJ1a by GST pull-down (Fig. 3, supplemental Fig. S3). Similar to other J domain-containing proteins, HSJ1a binds with HSP70 through its N-terminal J domain (Fig. 3*A*). Mutation of the conserved HPD motif (H31Q/D33N) completely abolished the interaction between them (8). Intriguingly, HSJ1a_JD cannot bind with the ATPase

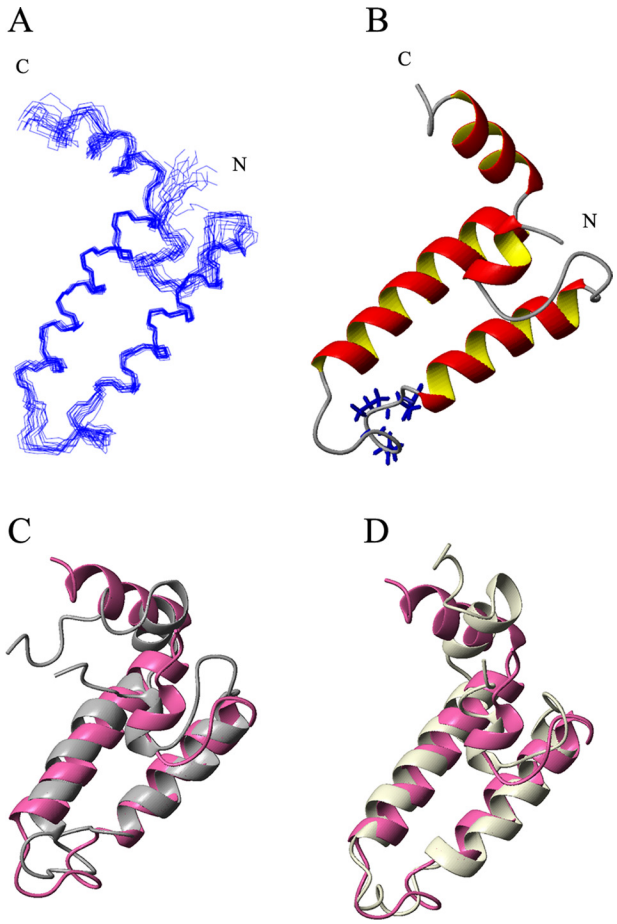

FIGURE 2. **Solution structure of the J domain of HSJ1a.** *A*, backbone superposition of 15 lowest energy structures of HSJ1a_JD (residues 1–71). *B*, ribbon representation of the structure of HSJ1a_JD. The structure is mainly comprised of four α-helices, helix-1 (residues 4–7), helix-2 (16–29), helix-3 (40–56), helix-4 (59–68), and a loop region (30–39) including the highly conserved ^{31}HPD motif highlighted in *blue*. *C*, comparison of the overall structures of HSJ1a_JD (*pink*) and HSP40_JD (PDB: 1HDJ, *gray*). *D*, comparison of the overall structures of HSJ1a_JD (*pink*) and DnaJ_JD (PDB: 1XBL, *light yellow*). The backbone RMSD of HSJ1a_JD with HSP40_JD is 4.5 Å and that with DnaJ_JD is 5.5 Å. The structures were generated by using MOLMOL.

domain (HSP1–384), as it is undetectable in the GST pull-down assay (Fig. 3*A*). The previous studies on prokaryotic DnaK and DnaJ have provided clues that the binding region of DnaK for DnaJ_JD is composed of the ATPase and substrate-binding domains (13, 29). However, under our experimental conditions, only a trace amount of HSP1–561 can be pulled down by HSJ1a_JD, whereas a large amount of HSP1–610 has been pulled down (Fig. 3*B*). As expected, both full-length HSP70 (1–641) and HSP1–631 are sufficient for binding with HSJ1a_JD (Fig. 3*C*). These data suggest that at least the N terminus of HSP70 including the ATPase domain and the substrate-binding β-subdomain is not sufficient for HSJ1a_JD binding.

To confirm this observation, we further studied the interactions by NMR titration, which is more sensitive than GST pull-down assay. ^{15}N-labeled HSJ1a_JD was titrated with an increasing amount of different C-terminally truncated HSP70 fragments. The HSQC spectra of ^{15}N-labeled HSJ1a_JD were obtained in the absence or presence of the HSP70 fragments. With titration of HSP70, the resonance peaks of the amides in HSJ1a_JD, such as His-31 and Asp-33, gradually become weak due to line broadening of the peaks originated from intermediate exchange in the NMR timescale regime (Fig. 4*A*), indicating that HSP70 binds with HSJ1a_JD. Based on the NMR titration data, we further obtained a profile of the relative peak heights of amides against the residue number of HSJ1a_JD during titration of HSP70 with molar ratios of 1:1, 1:2, and 1:3 (Fig. 4*B*). Almost all peak intensities corresponding to the J domain (1–71) significantly decrease with addition of HSP70 (Fig. 4*B*, supplemental Fig. S4). The peak intensities corresponding to the G/F-rich region also decrease but with a much less extent compared with those in the J domain. These signals originated from the G/F-rich region can be used as a well negative control. Upon HSP70 binding, there is no chemical-shift change observed, which is different from the result of DnaK and DnaJ in the previous study (13). As full-length HSP70, interaction of HSP1–610 shows a very similar profile on HSJ1a_JD (Fig. 4*C*, supplemental Fig. S5*A*). Titration of HSP1–595 causes a less extent of intensity decrease of HSJ1a_JD (Fig. 4*D*, supplemental Fig. S5*B*), being consistent with the result that HSP1–595 can be considerably activated by HSJ1a_JD (Fig. 1*B*). Also, titration of HSP1–561 causes a similar extent of intensity decrease with that of HSP1–595 (Fig. 4*E*, supplemental Fig. S5*C*), though this fragment is not considerably activated by HSJ1a_JD (Fig. 1*B*). This NMR titration experiment also corroborates the previous observation that HSP1–561 exhibits a weak interaction with HSJ1a_JD as evidenced by GST pull-down assay (Fig. 3*B*). As for titration with HSP1–384, the peak intensities of HSJ1a_JD remain almost unchanged (Fig. 4*F*, supplemental Fig. S5*D*), suggesting that HSP1–384 cannot interact with HSJ1a_JD. Similar result was also reported from surface plasmon resonance experiment that DnaJ failed to bind with DnaK1–385 but can bind with DnaK1–538 (27). Collectively, our results demonstrate that the N-terminal ATPase domain together with the substrate-binding β-subdomain of HSP70 is not sufficient for binding with the J domain of HSJ1a. Besides the substrate-binding domain, the C-terminal helices (residues 537–610) are also required for the effective binding and ATPase activation.

The C-terminal Helices Contribute to the Proper Conformation of HSP70 for HSJ1a_JD Binding and Activity Regulation—Considering that the C-terminal helices of HSP70 is important for HSJ1a binding and subsequent ATPase activity stimulation, we wonder how the C-terminal helical subdomain regulates the HSJ1a_JD binding and the ATPase cycle. We tested whether the C-terminal helices of HSP70 provide a binding site for HSJ1a_JD. The GST pull-down and NMR titration experiments suggest that the C-terminal subdomain does not directly bind with HSJ1a_JD (supplemental Fig. S6). So, we proposed that the C-terminal helices play a role in conformational switch of NBD and SBD domains and allosteric regulation during the ATPase cycle. We applied intrinsic fluorescence of tryptophan and quenching to probe the conformation of the N-terminal ATPase domain and the C-terminal helical subdomain. There are two natural Trp residues in wild-type HSP70; Trp-90 is resided in the N-terminal ATPase domain and Trp-580 in the C-terminal helical region. We generated HSP1–595 and HSP1–610 fragments each with a single-Trp residue by mutation of one Trp to Phe. The maximal wavelengths of fluorescence

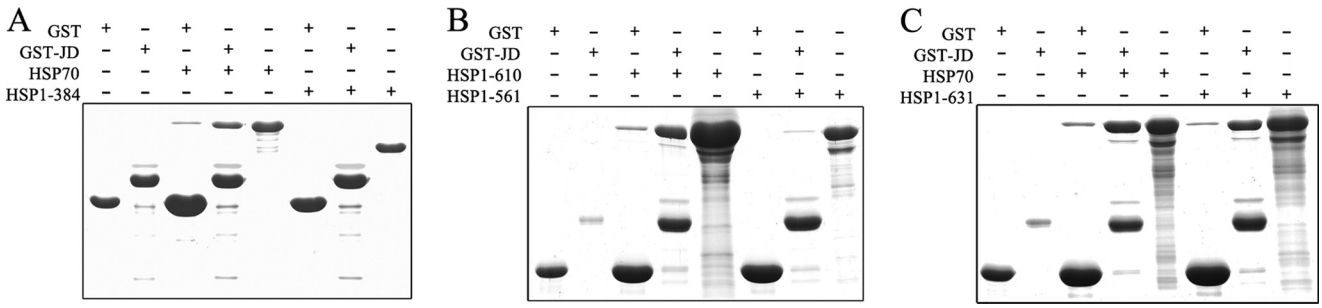

FIGURE 3. **Interaction of HSJ1a_JD with HSP70 and its fragments by GST pull-down experiments.** GST fused HSJ1a_JD was applied to pull down full-length HSP70 or HSP1–384 (*A*), HSP1–610 or HSP1–561 (*B*), and HSP1–631 (*C*). GST was set as a negative control.

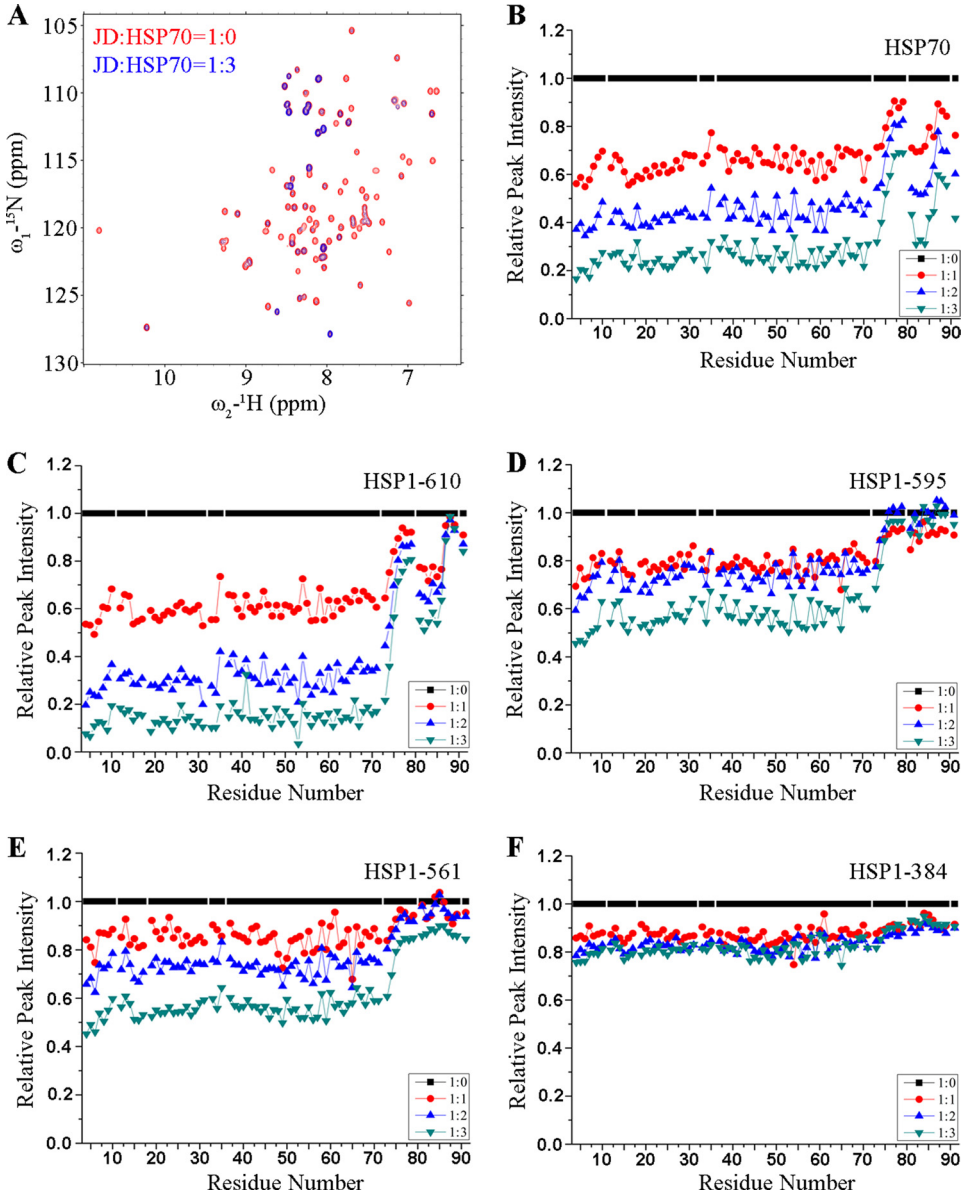

FIGURE 4. **NMR titrations showing the specific interaction between HSP70 and HSJ1a_JD.** *A*, overlay of the HSQC spectra of ^{15}N-labeled HSJ1a_JD (residues1–91,100 μM) (*red*) and addition of HSP70 at a molar ratio of 1:3 (*blue*). The remaining peaks after HSP70 titration are corresponding to the C-terminal flexible part of HSJ1a_JD (residues 75–91). *B*, plot of the relative peak heights of amides against the residue number of HSJ1a_JD in titration with HSP70. The peak heights were normalized as 1 for all peaks of free HSJ1a_JD except those for prolines and unassigned residues. *C*, as in *B*, titration with HSP1–610. *D*, as in (*B*), titration with HSP1–595. *E*, as in *B*, titration with HSP1–561. *F*, as in *B*, titration with HSP1–384.

emission are 347, 343, 340, and 342 nm for HSP1–595_W^{580}, HSP1–610_W^{580}, HSP1–595_W^{90} and HSP1–610_W^{90}, respectively (Fig. 5*A*). It suggests that the C-terminal helical subdomain in HSP1–610 is less exposed to solution than that in HSP1–595, whereas the ATPase domain where Trp90 is resided in HSP1–610 has a little more exposure than that in

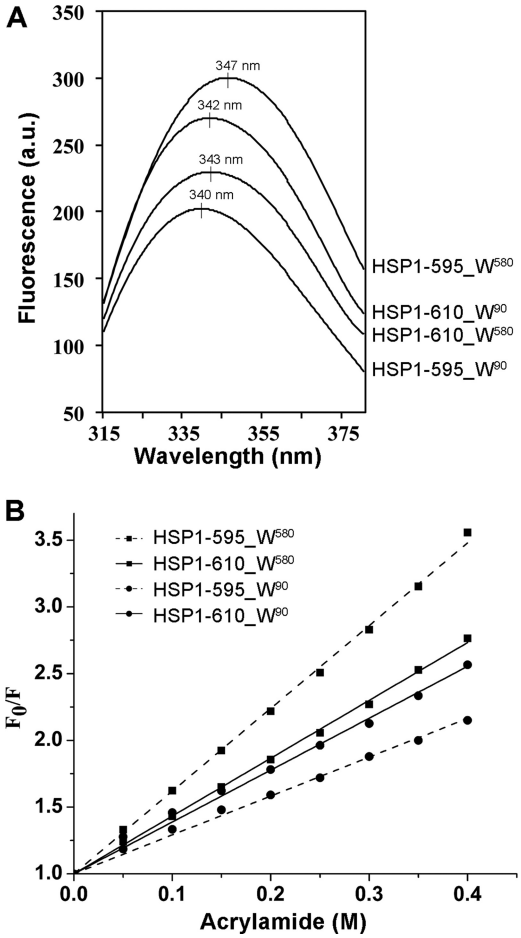

FIGURE 5. **Fluorescence study on the single-Trp HSP1–610 and HSP1–595 mutants.** *A*, intrinsic fluorescence emission of four types of mutants with different length and Trp location. These mutants include HSP1–595_W^{580}, HSP1–610_W^{580}, HSP1–595_W^{90}, and HSP1–610_W^{90} (~20 μM). Excitation wavelength was set at 295 nm, and the emission spectra were recorded from 315 to 380 nm. The maximal wavelength of each mutant is also indicated above the curve. *B*, acrylamide quenching of the intrinsic fluorescence. HSP1–610_W^{90} (*solid line*, *circle*); HSP1–610_W^{580} (*solid line*, *square*); HSP1–595_W^{90} (*dashed line*, *circle*); HSP1–595_W^{580} (*dashed line*, *square*). All the fluorescence intensities (*F*) were recorded at an emission of 340 nm. Three independent experiments were carried out for each mutant, and the average quenching constants (K_{SV}) were obtained by fitting with the Stern-Volmer equation. The K_{SV} values are 3.8 ± 0.1, 4.1 ± 0.2, 2.7 ± 0.2, and 6.1 ± 0.2 M^{-1} for HSP1–610_W^{90}, HSP1–610_W^{580}, HSP1–595_W^{90}, and HSP1–595_W^{580}, respectively.

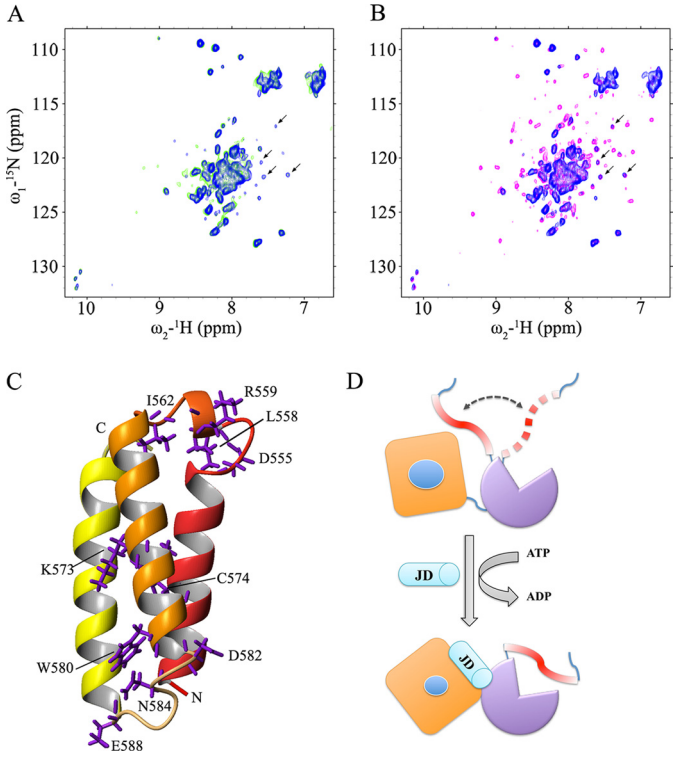

FIGURE 6. **HSJ1a_JD binding causes conformational change in the C-terminal helical subdomain of HSP70.** *A*, overlay of the HSQC spectra of segment 537–610 in HSP1–610 with Mg^{2+}-ATP (*green*) and addition of equal molar of HSJ1a_JD (*blue*). *B*, overlay of the HSQC spectra of segment 537–610 in HSP1–610 (*blue*) and ^{15}N-labeled peptide HSP537–610 (*pink*). The *arrows* indicate the "emerging" peaks. *C*, NMR solution structure of the C-terminal domain of HSP70 (HSP537–610) (PDB: 2LMG). The "emerging" peaks corresponding to the residues are indicated on the structure. *D*, schematic representation for the allosteric activation of HSP70 upon HSJ1_JD binding and ATP hydrolysis, highlighting the conformational fluctuation in the C-terminal helical subdomain. The ATPase, SBD-β, and SBD-α domains and ATP/ADP are indicated in *orange*, *purple*, *red*, and *blue*, respectively.

HSP1–595. Acrylamide quenching was also used to probe the Trp residues exposed in the protein environment (Fig. 5*B*, supplemental Fig. S7). By Stern-Volmer fitting (20), the quenching constants (K_{SV}) are 6.1, 2.7, 4.1, and 3.8 M^{-1} for HSP1–595_W^{580}, HSP1–595_W^{90}, HSP1–610_W^{580}, and HSP1–610_W^{90}, respectively. As expected, the Trp residue resided in the C-terminal helical region (Trp-580) is generally more exposed to solution than it in the ATPase domain (Trp-90). The conformational environments of the ATPase domain are slightly different in these two fragments due to the impact of the C-terminal helices. The Trp-90 residue in the HSP1–610 protein is more exposed than that in HSP1–595, suggesting that the last helix of HSP1–610 may have significant effect on the conformation of the ATPase domain. However, Trp-580 in HSP1–595 is also much more exposed than that in HSP1–610, suggesting that the environment of the Trp-580 residue is influenced by the helices of HSP1–610. So we presume that the C-terminal helices may cause dynamically structural switch of HSP70, providing a more dynamic and loose conformation for HSJ1a_JD binding. This subtle conformational fluctuation may be beneficial for HSJ1a_JD binding and subsequent ATPase activation.

HSJ1a_JD Binding Causes Conformational Change of the C-terminal Subdomain—To investigate conformational change of the C-terminal subdomain upon binding with HSJ1a_JD, we uniformly labeled the C-terminal segment (537–641) of HSP70 with ^{15}N by intein system for NMR experiments (31). In full-length HSP70, only the resonance peaks corresponding to the residues of the extremely flexible C-terminal part (611–641) were detected in the HSQC spectrum (data not shown). We then labeled the α-helical segment (537–610) in the context of HSP1–610 protein. As a result, not all the resonance peaks corresponding to the residues 537–610 were observed in the HSQC spectrum (Fig. 6*A*, *green*). Instead, there are three resonance peaks appeared in the indole amide region (~δ_H10.2 ppm), although only one Trp residue (Trp-580) in the segment 537–610, suggesting that at least three slow-exchange conformers exist in the Trp-580 region. This may attribute to the slow tumbling of the big size molecule of HSP1–610 and the slow-to-intermediate exchange of conformers in the α-helical

subdomain in the NMR timescale regime. However, upon addition of HSJ1a_JD, the intensities of some resonance peaks in the labeled α-helical region are increased, and these peaks return to appear in the HSQC spectrum (Fig. 6*A*, *blue*). These "emerging" peaks are well dispersed and correspond to the resonances of the peptide HSP537–610 (Fig. 6*B*, *pink*).

To recognize the peaks emerged in the HSQC spectrum of HSP1–610 upon HSJ1a_JD titration, we assigned the chemical shifts of the segment-labeled residues (537–610) by the ready assigned chemical shift pattern of the HSP537–610 peptide. These "emerging" peaks upon HSJ1a_JD binding are corresponding to Y545, D555, L558, K559, I562, K573, C574, W580, D582, N584, and E558. We also solved the solution structure of HSP537–610 by NMR techniques (supplemental Table S1). The structure (Fig. 6*C*) is almost the same as the previously reported crystal structure (PDB: 3LOF). Most of the residues that have significant changes in chemical shift or relaxation upon HSJ1a_JD binding are located in the linker regions between the helices, indicating that HSJ1a_JD binding may also cause conformational changes of the C-terminal helical domain of HSP1–610. Thus, the α-helical subdomain contains a cluster of conformers that are in fluctuation and exchange in the context of HSP1–610. The particular peaks emerged upon HSJ1a_JD binding and ATP hydrolysis may be caused by structural transformation of the C-terminal helical part from various fluctuating states associated with the N-terminal domains to the relatively stable SBD-β associated state (Fig. 6*D*). In other words, the C-terminal helical part of HSP70 is relatively fluctuating but somehow associates with the N-terminal domains of HSP70 in the absence of J-domain. Upon J-domain binding, the C-terminal helical part disassociates from the N-terminal NBD and transforms to a relatively stable state associating with the SBD (Fig. 6*D*). Taking together, this finding indicates that HSJ1a_JD binding may cause conformational switch of the C-terminal helical part of HSP70 and promote ATP hydrolysis by the N-terminal ATPase domain.

DISCUSSION

Structural Basis for the J Domains Binding with HSP70—There are many J-domain co-chaperones with different types of J-domains (32). DnaJ from *E. coli* is a type-I J protein, while both HSP40 and HSJ1a are eukaryotic type-II J proteins. Besides the J-domains, they also share a conserved G/F-rich region in their sequences. Our and previous studies have shown that the G/F-rich region is disordered in solution (9). The G/F-rich region of DnaJ is important for stabilizing substrate complex formation (28, 33). Our data suggest that the G/F-rich region of HSJ1a does not affect the interaction between HSJ1a and HSP70 and the ATPase activation (supplemental Figs. S2 and S4).

Though both the J domains and HSP70 proteins have high sequence conservation, their interaction modes are slightly different. For DnaJ, besides line broadening, some resonance peaks showed significant chemical-shift changes upon binding with DnaK, and the residues 2–35 were believed to form the surface for DnaK binding (13). In the case of HSJ1a_JD binding with HSP70, there exhibits the overall changes of peak intensities but no chemical-shift change, whether Mg^{2+} and ATP/ADP are present or not (supplemental Fig. S8). The structures of HSJ1a_JD and DnaJ_JD are something similar, except some difference lies in helix I and helix IV (Fig. 2*D*). This may provide structural basis for the different interaction modes of DnaK and HSP70 with their J-domain partners. However, the similarity of the J-domain structures of HSP40 and HSJ1a (Fig. 2*C*) suggests that they bind to HSP70 and stimulate ATPase activity with a very similar manner.

The C-terminal Helical Subdomain of HSP70 Is Crucial for HSJ1a_JD Binding and ATPase Activation—The binding sites of the J domains on HSP70 are controversial, although the ATPase domain of DnaK is evidenced for DnaJ binding (13). The crystal structure of bovine HSC70 complexed with the J domain of auxilin provides the structural basis for co-chaperone binding and regulation of HSP70 (14). It is not all inclusive, because in the structure the two molecules are covalently linked and HSC70 is C-terminally truncated. Actually, neither the physical interaction between the J domain and the separate ATPase domain nor the activation of the ATPase domain could be observed in our experiments (Figs. 1, 3, and 4) and in a number of literatures (27, 34). Our GST pull-down and NMR titration data suggest that the entire substrate-binding domain (SBD-β and SBD-α) is essential for J-domain binding. Compared with full-length HSP70 and HSP1–610, HSP1–561 with lack of the two C-terminal helices shows very low binding affinity to HSJ1a_JD. The C-terminal helical subdomain is likely to assist stabilizing the HSP70_JD complex and to enhance the binding affinity, but not to bind with the J domain directly (supplemental Fig. S6). The acrylamide quenching experiment supports this hypothesis, in which the conformation of the N-terminal ATPase domain is significantly affected by the last C-terminal helix (comparison between HSP1–595 and HSP1–610) (Fig. 5). Activation of the ATPase domain by HSJ1a_JD is also promoted by the C-terminal helices. As for HSP1–561, HSJ1a_JD cannot efficiently stimulate its ATPase activity, because it lacks the last two helices.

It is obvious that activation of HSP1–610 by the J domain of HSJ1a is more significant than that of full-length HSP70 (Fig. 1*B*). This leads us to consider the possible role of the flexible C-terminal end of HSP70 (including the EEVD motif) in the ATPase cycle, as in the case of DnaK (35). Based on the ATPase activities, it is presumptive that the flexible C-terminal end (611–641) of HSP70 may have a negative role in ATPase activation. Extensive studies should focus on the roles of the flexible C-terminal end in regulating the ATPase activation.

HSJ1a Binding Leads to Allosteric Activation of HSP70 through Conformational Change of the C-terminal Helices—Previous studies about the C-terminal helical subdomain mostly focused on the substrate binding (2, 36). Few attentions have been paid on the function of co-chaperone binding and the ATPase activation. According to the crystal structure of the substrate-binding domain of DnaK, only the first two helices are in the direct vicinity of the β-subdomain (2). Although DnaK and HSP70 are highly conserved analogues, their amino acid sequences of the last three helices are quite divergent. Since the J domain functions in stimulating ATP hydrolysis, it is likely that HSJ1a_JD binds either the nucleotide-free or ATP-bound form of HSP70. Recently, Senet *et al.* (3) have modeled the full-length structure of human inducible HSP70 based on

the crystal structures of its homologues. In the open model (ATP bound), the C-terminal helical subdomain is close to the ATPase domain but far from the substrate-binding domain (SBD-β). This provides a possibility for the alternative association between the ATPase domain and the helical subdomain (SBD-α) (Fig. 6*D*). Previous studies on the ATP-induced quenching of Trp-102 in DnaK suggest that docking of the ATP-bound ATPase domain onto the substrate-binding domain is gated by the C-terminal helical subdomain (37, 38). A recent study also suggests that the helical lid can swing around the hinge, locating the region of residues 525 to 540 by as much as 30 °C (26). These observations support our hypothesis that the C-terminal helical subdomain of HSP70 is fluctuating associated with the ATPase domain in the absence of J domain, and it will transform from the fluctuating states to a more stable state associating with the substrate-binding domain after J-domain binding or subsequent ATP hydrolysis (Fig. 6*D*).

Because of the high flexibility in its C terminus, it is often difficult to obtain the entire three-dimensional structure of the intact HSP70 protein. This study by biochemical approaches reveals that the C-terminal helical subdomain of HSP70, which acts as a switch for conformational fluctuation, is crucial for modulating J-domain interaction and allosteric activation, and subsequently regulating ATP hydrolysis. Extensive study will explore the role of the extreme end (EEVD sequence) in mediating interaction of HSP70 with the TPR domain-containing co-chaperones (30).

Accession Codes—Coordinates and structure factors have been deposited in the Protein Data Bank (PDB) with 2LGW for HSJ1a_JD and 2LMG for HSP537–610.

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