Direct interactions between molecular chaperones heat-shock protein (Hsp) 70 and Hsp40: yeast Hsp70 Ssa1 binds the extreme C-terminal region of yeast Hsp40 Sis1

Xinguo QIAN*, Wenbo HOU†, Li ZHENGANG* and Bingdong SHA†¹

*School of Life Sciences, University of Science and Technology of China, Hefei, People's Republic of China 230026, and †Department of Cell Biology, University of Alabama at Birmingham, 1918 University Blvd, Birmingham, AL 35294-0005, U.S.A.

Heat-shock protein 40 (Hsp40) enables Hsp70 to play critical roles in a number of cellular processes, such as protein folding, assembly, degradation and translocation *in vivo*. Hsp40 recognizes and binds non-native polypeptides and delivers them to Hsp70. Then Hsp40 stimulates the ATPase activity of Hsp70 to fold the polypeptides. By using yeast Hsp40 Sis1 and yeast Hsp70 Ssa1 as our model proteins, we found that the Sis1 peptide-binding fragment interacts directly with the full-length Ssa1 *in vitro*. Further studies showed that the C-terminal lid domain of Ssa1 could interact with Sis1 peptide-binding domain physically *in vitro*. The Sis1 peptide-binding fragment forms a stable complex with the Ssa1 C-terminal lid domain in solution. The interactions between these two proteins appear to be charge–charge interactions because high-ionic-strength buffer can dis-

sociate the complex. Further mapping studies showed that the Sis1 peptide-binding fragment binds the extreme C-terminal 15 amino acid residues of Ssa1. A flexible glycine-rich region is followed by these 15 residues in the Ssa1 primary sequence. Atomic force microscopy of the Sis1–Ssa1 complex showed that only one end of the Ssa1 lid domain binds the Sis1 peptide-binding-fragment dimer at the upper level of the huge groove within the Sis1 dimer. Based on the data, we propose an ‘anchoring and docking’ model to illustrate the mechanisms by which Hsp40 interacts with Hsp70 and delivers the non-native polypeptide to Hsp70.

Key words: AFM, atomic force microscopy, isothermal titration calorimetry, ITC, protein folding.

INTRODUCTION

The molecular chaperones are a large group of proteins that can recognize, bind and stabilize non-native polypeptides and facilitate protein folding [1,2]. A number of molecular chaperones were first identified as heat-shock proteins (Hsps) because these proteins are usually over-expressed when cells are challenged by heat shock or other cellular stress factors. Hsp70s play essential roles in cell physiology and are the best-studied family of molecular chaperones [1–3]. Hsp70s do not function alone. Hsp70 pairs with a specific Hsp40 to promote essential cellular functions such as protein folding, assembly, translocation and degradation of incorrectly folded polypeptides within cells [2–8]. At the molecular level, it is not well understood how Hsp40 interacts with Hsp70 and facilitates the protein refolding by Hsp70.

Hsp70 contains an N-terminal ATPase domain and a C-terminal peptide-binding domain [3,4,9,10]. In the crystal structure of the peptide-binding domain of *Escherichia coli* DnaK (the major Hsp70 in *E. coli*), complexed with its peptide substrate, two domains were identified: a β -domain and an α -domain [9]. The β -domain was composed of two layers of anti-parallel β -sheets and formed a peptide-binding groove, whereas the extreme C-terminal α -domain consisted of four α -helices and constituted a ‘lid’ domain to cover the peptide-binding groove [9].

All types of Hsp40 protein have an N-terminal J-domain (about 100 amino acid residues), which regulates the ATPase activity of Hsp70. The Hsp40 family has two subtypes: type I and type II. Type I Hsp40 protein has a zinc-finger motif adjacent to the J-domain and type II Hsp40 proteins do not. Both types of Hsp40 protein contain a C-terminal peptide-

binding fragment that may recognize and bind hydrophobic side chains of denatured polypeptides [11,12]. The J-domain within Hsp40 can transiently interact with the Hsp70 ATPase domain and stimulate the ATPase activity of Hsp70 [1,8,13]. It was proposed that Hsp40 binds non-native polypeptide first and then delivers the non-native polypeptide to Hsp70 for folding [11,13,14].

The direct interactions and complex formation between eukaryotic Hsp40 and Hsp70 have never been reported before. It has been shown that the extreme C-terminal four amino acid residues within human Hsp70, EEVD, play regulatory roles in Hsp40–Hsp70 interactions. Deletion of these four residues compromised the protein-refolding capability of human Hsp70 facilitated by human type II Hsp40 Hdj1 [15]. In order to investigate the mechanisms by which the Hsp40 peptide-binding fragment transfers non-native polypeptides to the Hsp70 peptide-binding domain, we utilized *Saccharomyces cerevisiae* Hsp40 Sis1 and *S. cerevisiae* Hsp70 Ssa1 as our model proteins to study the physical interactions between Hsp40 and Hsp70. Sis1 is an essential type II Hsp40 protein in *S. cerevisiae* [16] that can bind to ribosomes and which is required for the normal initiation of translation [17]. Ssa1 is one of the major yeast Hsp70 family members [18]. Sis1 can stimulate the ATPase activity of Ssa1 and facilitate the refolding of denatured protein with Ssa1 *in vitro* [12]. The crystal structure of the Sis1 C-terminal peptide-binding fragment (residues 171–352) has been determined [11]. The Sis1 C-terminal fragment forms a homodimer in crystal and in solution. Two hydrophobic depressions on the dimer surface were identified as the putative non-native polypeptide-binding sites. Mutations within the hydrophobic depressions will compromise the peptide-binding and molecular chaperone ability of Sis1 (results not

Abbreviations used: Hsp, heat-shock protein; ITC, isothermal titration calorimetry; AFM, atomic force microscopy; LB, Luria–Bertani.

¹ To whom correspondence should be addressed (e-mail sha@cmc.uab.edu).

Figure 1 Pull-down assays of yeast Hsp40 Sis1 peptide-binding fragment and yeast Hsp70 Ssa1 proteins

Both proteins are labelled with arrows. Lane 1, protein standards (from top to bottom: 97, 66, 45, 31, 21 and 14 kDa). Lane 2, purified Sis1 peptide-binding fragment. Lane 3, His-tagged Ssa1 lid domain bound to metal-chelating beads can pull down His-tag-free Sis1 peptide-binding fragment. The lower band represents the His-tagged Ssa1 lid domain and the upper band is the Sis1 peptide-binding fragment. Lane 4, His-tag-free Sis1 peptide-binding fragment was mixed with metal-chelating beads and washed with 10 mM Tris buffer (pH 7.9)/50 mM NaCl/50 mM imidazole. The beads were then subject to SDS/PAGE analysis. No apparent proteins could be detected, which indicates that the His-tag-free Sis1 peptide-binding fragment does not bind to metal-chelating beads. Lane 5, after His-tagged Ssa1 lid domain bound to metal-chelating beads pulled down the His-tag-free Sis1 peptide-binding fragment, the beads were washed with high-ionic-strength buffer [10 mM Tris buffer (pH 7.9)/0.2 M NaCl]. The washed beads were subjected to SDS/PAGE analysis. Lane 6, Ssa1 lid domain bound to metal-chelating beads.

shown). A large cleft generated by Sis1 dimer formation can accommodate the C-terminal peptide-binding domain of Hsp70 [11].

EXPERIMENTAL

Plasmids

The DNA encoding *S. cerevisiae* full-length Sis1 (residues 1–352) was cloned into vector pet15b (Novagen) using *NdeI* and *BamHI* (New England Biolabs) for protein expression. The Sis1 peptide-binding fragment (residues 171–352) was then cloned into pet15b using *NdeI* and *BamHI*. The lid domain region of Ssa1 (residues 524–642) was determined based on sequence alignment between Ssa1 and *E. coli* DnaK. A DNA fragment encoding the *S. cerevisiae* Ssa1 C-terminal lid domain (residues 524–642) was obtained by PCR and cloned into pet15b using *NdeI* and *BamHI*. A DNA fragment encoding the glycine-rich deletion mutation of the Ssa1 lid domain (LidΔG; the lid domain without the glycine-rich region, residues 609–627) was produced by PCR. The molecular masses of the Sis1 peptide-binding fragment and Ssa1 lid domain were approx. 20 and 12 kDa, respectively. Because the deletion site was close to the C-terminus (16 amino acid residues away from the C-terminus), a 5' primer and a long 3' primer that did not contain the nucleotide sequence of residues 609–627 were utilized to perform the PCR reactions. The PCR products were then ligated into pet15b using *NdeI* and *BamHI*. The deletion mutations were confirmed by subsequent DNA sequencing. The ATPase domain of Ssa1 (residues 1–386) was also cloned into pet15b.

Proteins

Yeast full-length Sis1 was over-expressed in *E. coli* strain BL21 (DE3). The limited proteolysis and protein purification of the Sis1 C-terminal peptide-binding fragment was performed as described previously [11]. The Sis1 C-terminal peptide-binding

fragment appeared to be free of contaminating proteins, as assessed by SDS/PAGE analysis. The purified Sis1 peptide-binding fragment was in its native state because it still retained peptide-binding activity and, furthermore, the purified protein can be crystallized [11]. A His-tagged Sis1 peptide-binding fragment can also be expressed using similar protocols, but with a lower yield.

The Ssa1 lid domain and the glycine-rich deletion mutation of the Ssa1 lid domain (LidΔG) were over-expressed in *E. coli* strain BL21(DE3). Luria-Bertani (LB) medium (10 ml) was inoculated using the transformed *E. coli* stocks. The cells were allowed to grow at 37 °C in a shaker for 12 h. Then the 10 ml of LB medium was used to inoculate 1 litre of LB medium. Isopropyl β-D-thiogalactoside (0.5 mM) was used to induce protein expression when the D_{600} value of the medium reached 0.6. The cells were harvested 3 h after induction. The *E. coli* cells from 1 l of medium were spun down by centrifugation and resuspended in 100 ml of 10 mM Tris buffer (pH 7.9)/150 mM NaCl. The cell walls and membranes were broken down by sonication. The debris and insoluble materials were centrifuged at 27200 g using a Beckman JA20 rotor and the supernatant was collected. Since the Ssa1 lid domain was histidine-tagged, it could be purified relatively easily using a metal-chelating column. The collected supernatant was pumped through the Ni²⁺-charged column and the protein-bound beads were washed thoroughly with 10 mM Tris buffer (pH 7.9)/0.5 M NaCl/50 mM imidazole to remove contaminating protein. The bound Ssa1 lid domain and LidΔG domain were then eluted with 10 mM Tris buffer (pH 7.9)/0.5 M NaCl/200 mM imidazole. The eluted protein was dialysed against 10 mM Tris buffer (pH 7.2)/50 mM NaCl. The N-terminal histidine-tags of the Ssa1 lid domain and LidΔG domain could be digested by thrombin treatment; 1 unit of thrombin (Sigma) was utilized for 1 mg of Ssa1 lid-domain protein. The digestion was carried out for 12 h at room temperature and stopped by the addition of 10 μl of 0.2 M PMSF, a protease inhibitor. The Ssa1 ATPase domain (residues 1–386) was also expressed in *E. coli* by use of a similar protocol. Yeast-expressed Hsp70 Ssa1 was obtained as described previously [12].

Pull-down assays

The pull-down assays were designed to detect the direct binding between the Sis1 peptide-binding fragment and wild-type or Ssa1 mutant proteins. The histidine-tagged Sis1 peptide-binding fragment and Ssa1 lid domain were expressed and purified as described above. About 0.5 mg of His-tagged Ssa1 lid domain was mixed with 50 μl of charged Ni²⁺-chelating resins in 10 mM Tris buffer (pH 7.9)/50 mM NaCl. Then about 1 mg of purified His-tag-free Sis1 peptide-binding fragment in 10 mM Tris buffer (pH 7.9)/50 mM NaCl was added to the resins. After being mixed gently for 20 min, the solution was centrifuged at 500 g for 5 min. The supernatant was discarded and the pelleted resins were washed extensively three times with 10 mM Tris buffer (pH 7.9)/50 mM NaCl/50 mM imidazole. The protein-bound resins were then placed in sample buffer for protein denaturation and SDS/PAGE analysis to reveal the binding between Sis1 and Ssa1. The pull-down assay between the His-tagged Sis1 peptide-binding fragment and wild-type His-tag-free Ssa1 was performed in a similar manner.

Gel-filtration chromatography

HPLC gel-filtration experiments were performed with a Superdex 200 gel-filtration column (Pharmacia) mounted on an AKTA HPLC system (Pharmacia). The column was pre-equilibrated

Figure 2 Gel-filtration chromatography studies of Sis1 peptide-binding fragment, Ssa1 lid domain and the complex of these two proteins

The top panel shows molecular standards. In the second panel, the major peak is the Sis1–Ssa1 complex and the minor peak is the excess Ssa1 lid domain. All proteins were in 10 mM Tris buffer (pH 7.9)/50 mM NaCl. The flow rate of the fluid was 1 ml/min. The gel-filtration studies were performed with an AKTA HPLC system (Pharmacia).

with 250 ml of appropriate filtered buffer (2 bed vol. of the column) before use. Gel-filtration standards were purchased from Bio-Rad.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out by use of an isothermal titration calorimeter from MicroCal (Northampton, MA, U.S.A.). All protein and buffer solutions were filtered and degassed before use. Sis1 peptide-binding fragment (protein concentration, 2.5 mg/ml) was added to the calorimetric cell and the Ssa1 lid domain or peptides L15, L7 and L4 (see the Results section for details) were injected into the cell with a 250 μ l injection syringe. Data on released heat were obtained by integrating the calorimetric output curves. Pure buffers were injected with the Sis1 peptide-binding fragment in control experiments.

The heat releases from the control experiments were subtracted from the experimental data for the Sis1 peptide-binding fragment before the data were utilized for K_d fitting. The K_d values were calculated by the software supplied with the calorimeter. All proteins and peptides were in 10 mM Tris buffer (pH 7.9)/50 mM NaCl.

Atomic force microscopy (AFM)

To reveal the nature of the Ssa1–Sis1 protein complex, the protein molecules were visualized by using an AFM system Nanoscope IIIA (Digital Instruments, Santa Barbara, CA, U.S.A.). All imaging was performed in tapping mode under solution in a fluid cell to minimize sample damage. An oxygen-sharpened silicon nitride tip with a spring constant of 0.58 N/m was used (Digital Instruments). The oscillation frequency was

chosen to be 30 kHz and the free amplitude of oscillation was set to 10–20 nm. The scan rate was 1.5 Hz. All the images were then processed using the software provided by the manufacturer.

AFM samples were prepared by a protein-binding technique [19]. Fresh protein (50 μ l; Sis1 or Sis1–Ssa1 complex) at about 1 μ g/ml in 10 mM Tris buffer (pH 7.0) was deposited on to a freshly cleaved mica surface. The surface was kept in a humid environment for about 20–30 min to avoid evaporation, allowing the positive protein molecules to bind to the negative surface. Plenty of Tris buffer was used to rinse the sample surface to remove unbound protein molecules. The sample was then transferred immediately to the fluid cell of the atomic force microscope to perform the scan.

RESULTS

Sis1 peptide-binding fragment binds directly to the Ssa1 lid domain and forms a complex through charge–charge interactions

We expressed and purified the yeast Hsp40 Sis1 peptide-binding fragment (residues 171–352), which was free of a histidine tag, and the N-terminal histidine-tagged yeast Hsp70 Ssa1 lid domain (residues 524–642). A pull-down assay was performed to test the interactions between these two proteins (Figure 1). The His-tagged Ssa1 lid domain was first bound to the metal-chelating resin, and then non-His-tagged Sis1 peptide-binding fragment was added to the solution to test the binding between two proteins. As indicated by the protein pull-down assay, the N-terminal His-tagged yeast Hsp70 Ssa1 lid domain bound physically to the yeast Hsp40 Sis1 peptide-binding fragment. These two proteins formed a stable complex with the resin in low-ionic-strength buffer [10 mM Tris buffer (pH 7.9)/50 mM NaCl]. However, the two proteins may dissociate in high-ionic-strength buffer [10 mM Tris buffer (pH 7.9)/0.2 M NaCl]. These data suggest that the Sis1 peptide-binding fragment binds the Ssa1 lid domain mostly through charge–charge interactions.

To study the interactions between the Sis1 peptide-binding fragment and Ssa1 lid domain further, we tried to create a complex of the two proteins *in vitro*. Purified Sis1 C-terminal peptide-binding fragment and Ssa1 lid domain were mixed together in Tris buffer (pH 7.2)/50 mM NaCl to constitute the Sis1 C-terminal fragment and Ssa1 lid-domain complex. The approx. molar ratio of Sis1 peptide-binding fragment to Ssa1 lid domain was about 2:3. Excess Ssa1 lid domain was added to the mixture to saturate the Sis1 C-terminal fragment in order to simplify purification of the complex. Because the complex of Sis1 peptide-binding fragment and Ssa1 lid domain had a much larger molecular mass than unbound Ssa1 lid domain, it was relatively easy to remove the unbound Ssa1 lid domain from the complex by gel-filtration chromatography. The Sis1–Ssa1 complex was purified by gel-filtration chromatography through a Superdex 200 column (Pharmacia) to remove the unbound Ssa1 lid domain. Gel-filtration studies showed that purified Sis1 peptide-binding fragment co-migrated with purified Ssa1 lid domain in solution (Figure 2). The apparent molecular mass of the Sis1–Ssa1 complex appeared to be approx. 60 kDa, as assessed from the elution time of the protein complex peak compared with the protein standards. The apparent molecular mass of the Ssa1 lid domain was shown to be approx. 20 kDa, indicating that the Ssa1 lid domain may form a dimer in solution (Figure 2).

To investigate the binding affinity between the Sis1 peptide-binding fragment and Ssa1 lid domain, we took advantage of the ITC technique, using a MicroCal isothermal titration calorimeter. Figure 3 shows the heat releases that occurred when Ssa1 lid-domain proteins were injected with the Sis1 peptide-binding

Figure 3 Calorimetric titration of the Sis1 peptide-binding fragment with the Ssa1 lid domain

Calorimetric output of 20 injections of the Ssa1 lid domain (0.11 mM) into the Sis1 peptide-binding fragment (0.028 mM) in 10 mM Tris buffer (pH 7.9)/50 mM NaCl.

fragment. The dissociation constant (K_d) between Sis1 and Ssa1 measured by ITC experiments was 17.1 μ M, indicating that the Sis1 peptide-binding fragment interacts with the Ssa1 lid domain with a relatively low binding affinity. The fitting of the ITC data indicated that the stoichiometry of the complex is one Sis1 peptide-binding-fragment dimer to one Ssa1 lid-domain molecule.

Sis1 peptide-binding fragment interacts with the extreme C-terminal 15 amino acid residues of the Ssa1 lid domain

The Ssa1 lid domain within the Sis1–Ssa1 protein complex was not stable. After being stored at 4 °C for 4 weeks, the Ssa1 lid

Figure 4 SDS/PAGE gel showing degradation of the Ssa1 lid domain within the complex

Lane 1, protein standards (from top to bottom: 97, 66, 45, 31, 21 and 14 kDa). Lane 2, purified fresh protein complex of the Sis1 peptide-binding fragment (top band) and Ssa1 lid domain by gel-filtration chromatography. Lane 3, purified fresh Ssa1 lid domain. Lane 4, degraded Sis1–Ssa1 protein complex after storage at 4 °C for 4 weeks. The degraded Sis1–Ssa1 complex was loaded on to a gel-filtration column and two peaks were observed. Lane 5 shows the SDS/PAGE result of the first peak, whose band position corresponds to the Sis1 peptide-binding fragment. The gel clearly shows that the Sis1 peptide-binding fragment no longer binds to the degraded Ssa1 lid domain. Lane 6, the second peak from the gel-filtration chromatography of the degraded Sis1–Ssa1 complex. The peak mainly contains degraded Ssa1 lid domain and a little contaminating Sis1 peptide-binding fragment.

Figure 5 Calorimetric titration of the Sis1 peptide-binding fragment with synthetic peptides

Calorimetric titration of Sis1 peptide-binding fragment (0.043 mM) with (top left panel) peptide L15 (0.7 mM), (bottom left panel) peptide L7 (0.33 mM), (top right panel) peptide L4 (0.8 mM) and (bottom right panel) Lid Δ G (0.082 mM). All proteins and peptides are in 10 mM Tris buffer (pH 7.9)/50 mM NaCl.

domain in the protein complex was degraded into a smaller protein fragment (Figure 4). Surprisingly, this degraded Ssa1 lid domain no longer bound to the Sis1 peptide-binding fragment, as revealed by gel-filtration studies. The degraded Ssa1 lid domain and Sis1 peptide-binding fragment migrated as two separate peaks from a gel-filtration column, and SDS/PAGE was used to show that the two peaks contained the Sis1 peptide-binding fragment and degraded Ssa1 lid domain (Figure 4). To characterize the degraded Ssa1 lid domain further, we conducted N-terminal sequencing and MS analysis. Five cycles of N-terminal sequencing showed that the N-terminal sequence of this protein was GSHME. Matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS analysis showed that the mass of the degraded Ssa1 lid domain was 10576.3 Da. Using these combined data, we concluded that the degraded Ssa1 lid domain was a truncated form lacking the extreme C-terminal 15 amino acid residues.

Because the degraded Ssa1 lid domain did not interact with the Sis1 peptide-binding domain, we reasoned that the protein motif containing the C-terminal 15 amino acid residues might be involved in binding to Sis1. To test this hypothesis, a peptide called L15, consisting of the 15 C-terminal amino acid residues of Ssa1, was synthesized (Research Genetics, Huntsville, AL, U.S.A.). The L15 peptide was purified further by using a C₈ reversed-phase column mounted on to an HPLC system. Subsequent ITC experiments showed that L15 bound to the Sis1 peptide-binding fragment with a K_d value of 13.6 μ M, which is very close to the binding affinity between the Sis1 peptide-binding fragment and Ssa1 lid domain (Figure 5, top left panel). This indicates that the Ssa1 lid domain interacts with the Sis1

peptide-binding fragment via the extreme C-terminal 15 residues. It is likely that other parts of Ssa1 do not play a role in binding the Sis1 peptide-binding fragment.

In the primary sequence of Ssa1, the extreme C-terminal 15 amino acid residues follow a glycine-rich region that covers about 20 residues (Figure 6). The glycine-rich region may serve as a flexible linker between the C-terminal 15 residues and the main part of Ssa1. To test whether the glycine-rich region of Ssa1 participates in binding to the Sis1 peptide-binding fragment, we constructed a glycine-region deletion mutant of the Ssa1 lid domain (Lid Δ G). ITC studies showed that the purified Lid Δ G protein bound the Sis1 peptide-binding fragment with a K_d value of 25.6 μ M, which is similar to that of the wild-type Ssa1 lid domain (Figure 5, bottom right panel). Thus the glycine-rich flexible linker of the Ssa1 lid domain may not be involved in interacting with the Sis1 peptide-binding fragment.

It has been shown that the extreme C-terminal four residues in human type II Hsp40 Hdj1, EEVD, play a regulatory role in facilitating Hsp70 chaperone activity [15]. Deletion of EEVD in the Hdj1 sequence abolished the regulatory role of Hdj1 in Hsp70 protein refolding [15]. The EEVD motif is also conserved in the Ssa1 primary sequence. To investigate whether a shorter peptide motif, containing the Ssa1 C-terminal sequence including EEVD, can interact with Sis1 peptide-binding fragment, we synthesized peptides L7 and L4 (Research Genetics). L7 represented the extreme C-terminal seven residues and L4 represented the extreme C-terminal four residues, EEVD. Both peptides of L7 and L4 were purified further by using a C₈ reversed-phase column. ITC studies showed that L7 and L4 did not interact with the Sis1 peptide-binding fragment (Figure 5, bottom left and top

Figure 6 Diagram of the domain structure of yeast Hsp70 Ssa1

The N-terminal ATPase domain is denoted by an open box and the C-terminal peptide-binding domain is represented by a hatched box. Two sub-domains within the peptide-binding domain, the peptide-binding groove and lid domain, are also specified. The amino acid sequence of the C-terminal region of the lid domain is shown. The glycine-rich flexible linker is shown in italic and the extreme C-terminal 15 amino acid residues (anchoring motif) are bold and underlined. Amino acid positions are also given.

Figure 7 AFM studies of yeast Hsp70 and Hsp40 complexes

Top left panel: AFM image of the Sis1 peptide-binding-fragment dimer. Top right panel: a surface potential image of the Sis1 peptide-binding-fragment dimer determined by GRASP [22]. Blue and red denote positively and negatively charged regions, respectively. The putative peptide-binding site and the groove are indicated. Domains I and II are also indicated. The elongated Sis1 peptide-binding-fragment monomer has a length of about 100 Å. Bottom panel: the AFM image of the Sis1 peptide-binding fragment and Ssa1 lid-domain protein complex. The Sis1 and Ssa1 moieties are labelled. The top left and bottom panels are shown at the same magnification, which is indicated by the scale axes in the top left panel.

Figure 8 The anchoring and docking model of how Hsp40 facilitates the delivery of non-native polypeptides to Hsp70

The grey object denotes Hsp40 and the white one represents Hsp70 in the ATP-bound state. The black line denotes the extended non-native polypeptide bound by the Hsp40 dimer through two hydrophobic peptide-binding sites on its surface. The grey curly line attached to Hsp40 represents the extreme C-terminal 15 amino acid residues (anchoring motif) and the flexible glycine-rich region that is in front of the anchoring motif.

right panels). Therefore, the EEVD motif by itself is not sufficient to sustain formation of the complex between the Ssa1 lid domain and Sis1 peptide-binding fragment. Our data showed that the extreme C-terminal 15 amino acid residues of Ssa1 are responsible for the binding to the Sis1 peptide-binding fragment.

The positioning of Sis1 and Ssa1 in the complex revealed by AFM studies suggests an 'anchoring and docking' model for Hsp40 to transfer non-native peptide to Hsp70 for folding

The structure of the Sis1–Ssa1 complex is of great interest in order to uncover the mechanisms by which Hsp40 delivers non-native polypeptides to Hsp70. AFM has been used to detect the interactions between *E. coli* GroEL and GroES *in vitro* [20]. In our studies, we utilized AFM to visualize the purified Sis1 peptide-binding fragment and the Sis1–Ssa1 complex in solution. Figure 7 shows the images of protein molecules of both the Sis1 peptide-binding fragment and the Sis1–Ssa1 complex. Figure 7 (top left panel) clearly demonstrates that the Sis1 peptide-binding-fragment dimer contains a large groove within the molecule. Indeed, the crystal structure of the Sis1 peptide-binding domain showed that the Sis1 homodimer forms a huge groove between the two monomers [11]. Therefore, the visualized shape of Sis1 is in good agreement with the crystal structure solved by X-ray diffraction (Figure 7, top right panel), which indicates the reliability of the AFM experiments.

We then performed AFM studies on the protein complex of the Sis1 peptide-binding fragment and Ssa1 lid domain to investigate how these two proteins interact. The molecular conformation of the Sis1–Ssa1 complex is shown in Figure 7 (bottom panel). Although the resolution of this image is not as high as that of Figure 7 (top left panel), due to the possible impurity effect, Sis1 can still be recognized in the complex by its

unique groove feature (Figure 7, bottom panel). The Ssa1 lid domain is shown as a rod-shaped object in the AFM image, which is consistent with X-ray crystallographic studies of the Hsp70 DnaK lid domain. AFM images of the Sis1–Ssa1 complex showed that only one end of the Ssa1 lid domain attaches with the Sis1 peptide-binding fragment (Figure 7, bottom panel). This is consistent with our finding that only the C-terminal 15 residues of Ssa1 are involved in binding to the Sis1 peptide-binding fragment.

On the basis of crystal structures, it has been proposed that the Sis1 and Ssa1 proteins may dock to carry out the non-native polypeptide transfer [11]. Our data suggest that the Ssa1 C-terminal 15 residues and adjoining glycine-rich flexible linker may function as an anchor to direct the Ssa1 protein in docking with the Sis1 protein. Therefore, we propose an 'anchoring and docking' model to illustrate the mechanisms by which Hsp40 interacts with and delivers non-native polypeptides to Hsp70 (Figure 8).

DISCUSSION

We have discovered, by use of pull-down assays, that the yeast Hsp40 Sis1 peptide-binding fragment could interact physically with the yeast Hsp70 Ssa1 C-terminal lid domain through charge–charge interactions. The protein complex of the Sis1 peptide-binding fragment and Ssa1 lid domain was constituted and purified. The binding region of Ssa1 was mapped to the extreme C-terminal 15 amino acid residues by use of ITC. A glycine-rich region (about 20 residues) in front of the Ssa1 15 C-terminal amino acid residues may not participate in binding to the Sis1 peptide-binding fragment and instead function as a flexible linker. AFM studies showed that one end of the Ssa1 lid-domain molecule attaches to the Sis1 peptide-binding-fragment dimer at the upper part of the large groove that is formed by Sis1 domain II. The main body of the Ssa1 lid domain stays adjacent to the Sis1 groove. Based on the data obtained, we hypothesize an anchoring and docking model to demonstrate the mechanisms by which Hsp40 delivers non-native polypeptides to Hsp70 for folding.

It has been reported that the Hsp70 peptide-binding domain prefers to bind non-native peptides in an extended conformation [9]. Because the two peptide-binding sites are separated by approx. 30 Å (1 Å = 0.1 nm), the Sis1 dimer may stretch out the non-native polypeptides through its two peptide-binding sites for subsequent Hsp70 folding [11]. The yeast Hsp70 Ssa1 C-terminal 15 amino acid residues form an anchoring motif that may direct Hsp70 to dock with Hsp40 in order to receive non-native polypeptides. The glycine-rich region in front of the anchoring motif of Ssa1 may form a very flexible linker between the main body of Ssa1 and the anchoring motif. The glycine-rich linker may provide Ssa1 with substantial flexibility to recognize and bind the non-native polypeptides extended by Sis1, whereas the C-terminus of Ssa1 is anchored to the upper part of the Sis1 groove. Sequence alignment shows that the glycine-rich linker is conserved among all eukaryotic Hsp70s [15]. The extended non-native polypeptide may expose its hydrophobic side chains for subsequent Hsp70 binding. All together, the anchoring and docking model may elucidate the mechanism by which Hsp40 efficiently facilitates non-native polypeptide transfer to Hsp70 (Figure 8).

AFM studies of the Sis1–Ssa1 complex also indicate that the Ssa1 anchoring motif (the extreme C-terminal 15 residues) interacts with the Sis1 peptide-binding fragment on the upper part of the groove (Figure 7, bottom panel). The Ssa1 lid domain stays adjacent to the groove while the Ssa1 anchoring motif

attaches to the Sis1 peptide-binding fragment at the upper part of the groove. This will leave sufficient space for Sis1 to stretch out the non-native polypeptide and to deliver it to the Ssa1 peptide-binding domain. The glycine-rich linker in front of the anchoring motif in Ssa1 may provide the flexibility needed for Ssa1 to accommodate the stretched out non-native polypeptides into the Ssa1 peptide-binding groove (Figure 8). Genetic studies have shown recently that the ability of yeast Hsp40s (Sis1 and Ydj1) to bind unfolded protein substrates is an essential function *in vivo* [21]. The model proposed here demonstrates the mechanism by which Hsp40 delivers denatured peptides to Hsp70 and suggests that Hsp40 plays essential roles in cell physiology.

It remains unclear how Ssa1 and Sis1 dissociate from each other after non-native polypeptide transfer. ATP hydrolysis may generate conformational changes within Hsp70 Ssa1 and close the lid domain on the peptide-binding groove. This may cause the dissociation of Ssa1 and Sis1. Our ITC studies showed that the Sis1 peptide-binding fragment binds the Ssa1 lid domain with relatively low affinity (K_d approx. 20 μ M). This may ensure dissociation of the protein complex after peptide delivery.

We wish to thank Dr Elizabeth Craig for providing us with the Ssa1 cDNA. This work was supported by National Institutes of Health grant R01 DK56203, Howard Hughes Memorial Institute, the American Heart Association and NASA.

REFERENCES

- Gething, M. J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* (London) **355**, 33–45.
- Craig, E., Weissman, J. S. and Horwich, A. L. (1994) Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. *Cell* **78**, 365–372.
- Hartl, F. U. (1996) Molecular chaperones in cellular protein folding. *Nature* (London) **381**, 571–580.
- Bukau, B. and Horwich, A. L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351–366.
- Georgopoulos, C. (1992) The emergence of the chaperone machines. *Trends Biochem. Sci.* **17**, 295–299.
- Flynn, G., Pohl, J., Flocco, T. and Rothman, J. (1991) Peptide-binding specificity of the molecular chaperone BiP. *Nature* (London) **353**, 726–730.
- Ungermann, C., Neupert, W. and Cyr, D. M. (1994) The role of Hsp70 in conferring unidirectionality on protein translocation into mitochondria. *Science* **266**, 1250–1253.
- Rothman, J. E. (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell* **59**, 591–601.
- Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E. and Hendrickson, W. A. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606–1614.
- Flaherty, K. M., DeLuca-Flaherty, C. and McKay, D. B. (1990) Three-dimensional structure of the ATPase fragment of a 70 K heat-shock cognate protein. *Nature* (London) **346**, 623–628.
- Sha, B. D., Lee, S. and Cyr, D. M. (2000) The crystal structure of the peptide-binding fragment from the yeast Hsp40 protein Sis1. *Structure* **8**, 799–807.
- Lu, Z. and Cyr, D. M. (1998) Protein folding activity of Hsp70 is modified differentially by the Hsp40 cochaperones Sis1 and Ydj1. *J. Biol. Chem.* **273**, 27824–27830.
- Hohfeld, J., Minami, Y. and Hartl, F. U. (1995) Hip, a novel cochaperone involved in the eukaryotic hsc70/hsp40 reaction cycle. *Cell* **83**, 589–598.
- Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K. and Hartl, F. U. (1992) Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* (London) **356**, 683–689.
- Freeman, B. C., Myers, M. P., Schumacher, R. and Morimoto, R. I. (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with Hdj-1. *EMBO J.* **14**, 2281–2292.
- Luke, M. M., Sutton, A. and Arndt, K. T. (1991) Characterization of Sis1, a *S. cerevisiae* homologue of bacteria dnaJ proteins. *J. Cell Biol.* **114**, 623–638.
- Zhong, T. and Arndt, K. T. (1993) The yeast Sis1 protein, a DnaJ homolog, is required for the initiation of translation. *Cell* **73**, 1175–1186.
- Lopez-buesa, P., Pfund, C. and Craig, E. A. (1998) The biochemical properties of the ATPase activity of a 70 kDa heat shock protein are governed by the C-terminal domains. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15253–15258.
- Radmacher, M., Fritz, M., Hansma, H. G. and Hansma, P. K. (1994) Direct observation of enzyme activity with atomic force microscopy. *Science* **265**, 1577.
- Viani, M. B., Pietrasanta, L. I., Thompson, J. B., Chand, A., Gebeshuber, I. C., Kindt, J. H., Richter, M., Hansma, H. G. and Hansma, P. K. (2000) Probing protein-protein interactions in real time. *Nat. Struct. Biol.* **7**, 644–647.
- Johnson, J. L. and Craig, E. A. (2001) An essential role for the substrate-binding region of Hsp40s in *Saccharomyces cerevisiae*. *J. Cell Biol.* **152**, 851–856.
- Nicholls, A., Sharp, K. A. and Honig, B. (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11**, 281–296.

Received 8 August 2001/1 October 2001; accepted 25 October 2001