Crystal structure of yeast Sis1 peptide-binding fragment and Hsp70 Ssa1 C-terminal complex

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Heat shock protein (Hsp) 40 facilitates the critical role of Hsp70 in a number of cellular processes such as protein folding, assembly, degradation and translocation *in vivo*. Hsp40 and Hsp70 stay in close contact to achieve these diverse functions. The conserved C-terminal EEVD motif in Hsp70 has been shown to regulate Hsp40–Hsp70 interaction by an unknown mechanism. Here, we provide a structural basis for this regulation by determining the crystal structure of yeast Hsp40 Sis1 peptide-binding fragment complexed with the Hsp70 Ssa1 C-terminal. The Ssa1 extreme C-terminal eight residues, G⁶³⁴PTVEEVD⁶⁴¹, form a β -strand with

the domain I of Sis1 peptide-binding fragment. Surprisingly, the Ssa1 C-terminal binds Sis1 at the site where Sis1 interacts with the non-native polypeptides. The negatively charged residues within the EEVD motif in Ssa1 C-terminal form extensive charge–charge interactions with the positively charged residues in Sis1. The structure-based mutagenesis data support the structural observations.

Key words: crystal structure, Hsp40, Hsp70, molecular chaperone, Sis1, Ssa1.

INTRODUCTION

Molecular chaperones are a large group of proteins that recognize, bind and stabilize non-native polypeptides and facilitate protein folding [1–3]. A number of molecular chaperones were first identified as Hsps (heat shock proteins) because these proteins are usually over-expressed when the cells are challenged by heat shock or other cellular stress factors. The Hsp70 family plays an essential role in cell physiology and is the best studied of the molecular chaperones [1–3]. Members of the Hsp70 and Hsp40 (DnaJ-like) families function in specific pairs that form transient complexes with non-native regions of polypeptides to promote the protein folding, assembly and transport within the cell [1–6].

All types of Hsp40s contain an N-terminal J-domain that can stimulate the ATPase activities of Hsp70. Both type I and type II Hsp40s have a peptide-binding fragment located at the C-terminal of the protein. In both type I and type II Hsp40s, their J-domains are connected to the peptide-binding fragments via a glycine-/phenylalanine-rich linker. However, type I Hsp40s such as *Escherichia coli* DnaJ, yeast Ydj1 and human Hdj-2 contain two zinc-finger-like motifs within their primary sequences, which are located between the J-domain and the C-terminal peptide-binding fragment, whereas type II Hsp40s such as yeast Sis1 and human Hdj1 do not [7–9]. It was proposed that Hsp40 first binds non-native polypeptide and then delivers it to Hsp70 for folding [1,10,11]. The ability to bind non-native polypeptides for the cytosolic Hsp40 is an essential function *in vivo* [12].

The crystal structure of the peptide-binding fragment of Sis1, a type II yeast Hsp40, was determined previously [13]. The crystal structure revealed that Sis1 functions as a homodimer with a U-shaped molecular structure. A large cleft forms between the two elongated Sis1 monomers. Each Sis1 monomer contains domains I, II and a short C-terminal dimerization motif. Both domain I and

II have a core formed by a major β -sheet and a minor β -sheet that are connected by a short helix. A hydrophobic depression is located on the molecular surface of domain I of the Sis1 peptide-binding fragment monomer. The Sis1 dimer may utilize the two hydrophobic depressions as peptide-binding sites to interact with the non-native polypeptides through hydrophobic interactions [13,14]. The crystal structure of the peptide-binding fragment of type I Hsp40 Ydj1 complexed with its peptide substrate was also determined in our laboratory [15,16]. Domains I and III of Ydj1 resemble the structure of domain I and II of Sis1, while two zinc-finger motifs form domain II of Ydj1. In the crystal structure of Ydj1 complexed with its peptide substrate, the peptide substrate binds to domain I of Ydj1 and forms a β -strand with the Ydj1 molecule. The side chains of the peptide substrate make extensive hydrophobic interactions with a hydrophobic pocket located on domain I of Ydj1.

Hsp70 contains an N-terminal ATPase domain and a C-terminal peptide-binding domain [1,5]. In the crystal structure of *E. coli* Hsp70 DnaK peptide-binding domain complexed with its peptide substrate, two domains were identified, a β -domain and an α -domain [17]. The β -domain is composed of two layers of anti-parallel β -sheets and forms a peptide-binding groove, while the extreme C-terminal α -domain consisted of four α -helices that constitute a ‘lid’ domain to cover the peptide-binding groove [17].

It has been shown previously, that the C-terminal four EEVD residues within human Hsp70 play regulatory roles in Hsp40/Hsp70 function [4]. Deletion of these four residues compromises the protein refolding capability of human Hsp70 facilitated by human type II Hsp40 Hdj1 [4,6]. The direct interactions between yeast Hsp40 Sis1 peptide-binding fragment and the C-terminal of yeast Hsp70 Ssa1 have been characterized and the protein complex between Sis1 peptide-binding fragment and the Ssa1 lid

Abbreviations used: APS, advanced photon source; Hsp, heat shock protein; HOP, Hsp70 and Hsp90 organizing protein; ITC, isothermal titration calorimetry; MAD, multiple anomalous dispersion; SER-CAT, Southeast regional collaborative access team; TPR tetratricopeptide.

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The co-ordinates for Sis1–Ssa complex have been deposited in the Protein Data Bank under accession number 2B26.

domain has been reconstituted [6,18]. The J-domain of Hsp40 cannot form a stable complex with Hsp70. In the present report, we show the crystal structure of Sis1 peptide-binding fragment complexed with the C-terminal of Hsp70 Ssa1. The Ssa1 extreme C-terminal eight residues, G⁶³⁴PTVEEVD⁶⁴¹, form a β -strand with domain I of Sis1 peptide-binding fragment. The Ssa1 C-terminal binds Sis1 at the site where Sis1 interacts with the non-native polypeptides. Binding of the C-terminal of Ssa1 to Sis1 causes conformational changes in Sis1 that twist domain I further away from the main body of the Sis1 protein. Structure-based mutagenesis indicates that the charge-charge interactions between the EEVD motif in the C-terminal of Ssa1 and Sis1 are critical for binding of Sis1 to Ssa1.

EXPERIMENTAL

Proteins

The crystallization of the protein complex of Sis1–Ssa1 has been described previously [6,19]. The Sis1 peptide-binding fragment (residues 171–352) and Ssa1 lid domain (residues 524–641) were utilized in reconstituting the protein complex. The structure-based mutagenesis was carried out using the QuikChange[®] kit (Stratagene). The mutant Sis1 proteins were purified using the same protocol as the wild-type Sis1.

Crystallization of the Sis1–Ssa1 complex

The crystallization and data collection were described previously for the Sis1–Ssa1 complex [19]. Briefly, the complex was crystallized by using the hanging drop vapour diffusion method, with the mother liquid containing 100 mM Tris/HCl, pH 7.0 and 42.5 % (w/v) NH₄SO₄. The crystals belong to the space group of P4₁2₁2 with the cell parameters of $a = 112.17$ Å (1 Å = 0.1 nm) and $c = 171.31$ Å. The MS and SDS/PAGE (13 % gels) analysis of the complex crystals indicated that the crystals contained Sis1 peptide-binding fragment and Ssa1 lid domain. The ITC (isothermal titration calorimetry) studies indicated that a single Sis1 dimer bound to a single Ssa1 lid domain monomer.

Structure determination and refinement

The MAD (multiple anomalous dispersion) data for the Sis1–Ssa1 complex crystals were collected at APS (advanced photon source) SER-CAT (Southeast Regional Collaborative Access Team) beamline. Se-Met Sis1 peptide-binding fragment was used to reconstitute the complex. The MAD method was utilized to determine the structure of Sis1–Ssa1 complex. The selenium atoms were found and phases were calculated using the program SOLVE. The RESOLVE program was utilized to carry out density modification and solvent flattening [20]. Three Sis1 peptide-binding fragment molecules A, B and C were modelled into the electron density map using program O [21]. Monomers A and B form a homodimer while C forms a homodimer with a symmetry-related monomer. In molecule A, the majority of Sis1 peptide-binding fragment (residues 180–349) can be modelled into the electron density with the exception of the residues 232–246. The extreme C-terminal eight residues G⁶³⁴PTVEEVD⁶⁴¹ of the Hsp70 Ssa1 lid domain are visible in molecule A in the electron density map. The other part of Ssa1 lid domain is missing in the electron density map, possibly due to the flexibility of the molecule (see Figure 1). In molecule B, most of the Sis1 peptide-binding domain (residues 180–349) is visible in the electron density map, with the exception of residues 201–214. However, the electron density map for the Ssa1 C-terminal in molecule B is not clear enough to allow unambiguous modelling. In molecule C, only domain II and the C-terminal dimerization motif (residues

238–349) of Sis1 are visible in the electron density map. The Ssa1 extreme C-terminal eight amino acid residues G⁶³⁴PTVEEVD⁶⁴¹ associated with molecule A were also modelled into the electron density map. The model was then refined using the CNS program against the 3.2 Å data collected at APS [22]. One cycle of temperature annealing (at 2000 K) and six cycles of positional refinement were then carried out. Restrained individual B-factor refinement was not performed until the last cycle. After each cycle of refinement, the model was manually rebuilt according to the resultant $2F_o - F_c$ and $F_o - F_c$ maps. No water molecules were added into the final electron density map due to the low-resolution limit for the data set. The refinement gave reasonable root mean square deviation from the ideal geometry at this resolution (Table 1). A Ramachandran plot of the final model (using the Proboty program; <http://kinemage.biochem.duke.edu>) revealed that 87.7 % of the residues in the structure were in the favoured regions and 97.2 % of the residues were in allowed regions [23].

ITC assay

Measurement of binding between Sis1 and the Sis1 mutants with Ssa1 lid domain was carried out by use of an isothermal titration calorimeter (MicroCal) at room temperature (22 °C). Sis1 (or Sis1 mutants) as well as the Ssa1 lid domain were dialysed against the same buffer (30 mM Hepes, pH 7.5, 50 mM KCl and 10 mM MgCl₂). The calorimetric cell was filled with Sis1 (or Sis1 mutants; at a concentration of 0.03 mM) and the Ssa1 lid domain (0.12 mM) was injected into the cell with a 250 μ l syringe. The released heat was measured by integrating the calorimetric output curves. Pure buffers were injected into the calorimetric cell as control experiments. The heat releases from the control experiments were subtracted from the experimental data before they were used for K_d fitting. The K_d values were calculated using the software supplied with the calorimeter.

Co-ordinates

The co-ordinates and structure factors of the Sis1–Ssa1 complex have been deposited in the Protein Data Bank with the accession number 2B26.

RESULTS

Structure determination of the Sis1 peptide-binding fragment complexed with the Ssa1 C-terminal lid domain

We expressed and purified the yeast type II Hsp40 Sis1 peptide-binding fragment (residues 171–352) and the yeast Hsp70 Ssa1 lid domain (residues 524–641). We then constituted and crystallized the protein complex between Sis1 peptide-binding fragment and Ssa1 lid domain [6,19]. The crystal structure of the Sis1 peptide-binding fragment complexed with the Ssa1 C-terminal lid domain was determined to 3.2 Å resolution by the MAD method using the Se atoms as the anomalous scattering centres (Table 1). The resultant electron density map from the MAD phasing was readily traceable. The extreme C-terminal eight residues G⁶³⁴PTVEEVD⁶⁴¹ of Hsp70 Ssa1 lid domain are visible in the electron density map. The other part of Ssa1 lid domain is missing in the electron density map probably due to the flexibility of the molecule (Figure 1). It has been shown that the extreme C-terminal seven residues PTVEEVD of yeast Hsp70 Ssa1 is responsible for interaction with Hsp40 Sis1 [6]. Our complex structure is consistent with this data.

Sis1 and Ssa1 interactions

The Ssa1 C-terminal eight residues, G⁶³⁴PTVEEVD⁶⁴¹, form an anti-parallel β -strand with the B2 in the minor β -sheet of Sis1

Figure 1 Crystal structure of the Sis1-Ssa1 complex

(A) Ribbon drawing of the Sis1-Ssa1 complex dimer [25]. Monomer A of Sis1 is shown in silver and monomer B is shown in gold. The C-terminal of Ssa1 is shown in red. The two monomers A and B are related by a vertical two-fold axis. Domain I and II of Sis1 are labelled. (B) A view of Sis1 dimer after it is rotated 90° along the vertical axis from the orientation shown in (A). (C) The $2F_o - F_c$ map of the molecule A of the Sis1-Ssa1 complex at the interaction site between Sis1 and the Ssa1 C-terminal. Some of the residues of the Ssa1 C-terminal are labelled. The C α backbone of Ssa1 is shown in yellow and the C α backbone of Sis1 is shown in red.

domain I (Figure 1). The minor β -sheet of the domain I of Sis1 contains three β -strands after binding to the Ssa1 C-terminal. Of the eight residues of the Ssa1 C-terminal, the main-chain atoms of the five residues TVEEV form the typical β -sheet hydrogen-bond networks with the backbone atoms of residues 200–204 of Sis1 B2.

The side chains of the C-terminal of Ssa1 make extensive interactions with Sis1 molecules (Figure 2). The three negatively charged residues Glu⁶³⁸, Glu⁶³⁹ and Asp⁶⁴¹ within the EEVD motif form a number of salt bridges with the positively charged residues from Sis1. Glu⁶³⁸ of Ssa1 forms charge-charge interactions with Lys²⁰² and Lys²¹⁴ from Sis1. Glu⁶³⁹ of Ssa1 interacts with Lys¹⁹⁹ and Lys²⁵⁶ of Sis1 through charge-charge interactions. Asp⁶⁴¹ of Ssa1 forms salt bridges with Lys¹⁹⁹ of Sis1 (Figure 2). The extensive charge-charge interactions between Ssa1 and Sis1 explain the observation that the Ssa1-Sis1 complex is sensitive to high salt concentration [6].

In addition to the charge-charge interactions between Ssa1 and Sis1, the side chain of Val⁶³⁷ from the Ssa1 C-terminal eight residues (G⁶³⁴PTVEEV⁶⁴¹) makes hydrophobic interactions with the Sis1 peptide-binding depression. The crystal structure of the yeast Hsp40 Sis1 revealed a hydrophobic depression located on domain I that may be responsible for binding the hydrophobic side chains of the non-native polypeptides [13]. The side chain of Val⁶³⁷ of Ssa1 occupies this hydrophobic depression of Sis1 and makes hydrophobic interactions with Sis1. The side chain of Pro⁶³⁵ also makes hydrophobic interactions with the Sis1 peptide-binding depression located on domain I (Figure 3A).

Comparison of Sis1-Ssa1 complex structure with Ydj1-peptide-substrate complex structure

The yeast Hsp70 Ssa1 C-terminal binds type II Hsp40 Sis1 at its peptide-binding depression located at domain I (Figure 1). We

Table 1 Statistics for MAD data collection and structure determination

MAD phasing statistics were performed with a figure of merit of 0.435. Numbers in parentheses are for the outer resolution shell.

(a) Data collection

Parameter	Peak	Edge	Remote I	Remote II
Resolution (Å)	3.2	3.2	3.2	3.2
R_{sym}	0.067 (0.329)	0.066 (0.333)	0.072 (0.392)	0.074 (0.385)
Completeness (%)	89.8% (76.0%)	90.6% (76.8%)	91.1% (79.6%)	90.1% (73.1%)
$\langle I/\sigma \rangle$	18.1 (5.4)	18.2 (5.9)	18.2 (6.0)	17.3 (5.7)
Redundancy	3.36	3.38	3.43	3.82

(b) Refinement

Parameter	Value
Resolution range (Å)	30.0–3.2
Number of used reflections	17558 (1754 used for R_{free} calculation)
R_{factor} (%)	27.6 (36.8 for outer resolution shell)
R_{free} (%)	33.9 (41.5 for outer resolution shell)
Number of model atoms	3435 (0 water molecules)
Root mean square deviations from ideality	
Bond lengths (Å)	0.008
Bond angles (°)	1.486
Impropers (°)	1.032
Dihedrals (°)	25.86

Figure 2 Charge-charge interactions between the EEVD motif of Ssa1 and Sis1

The Ssa1 C-terminal is shown in red and Sis1 is shown in silver. Lys¹⁹⁹, Lys²⁰², Lys²¹⁴ and Lys²⁵⁶ from Sis1, and Glu⁶³⁸, Glu⁶³⁹ and Asp⁶⁴¹ from the Ssa1 EEVD motif are labelled in the Figure.

have determined previously the crystal structure of yeast type I Hsp40 Ydj1 complexed with its peptide substrate GWLYEIS [15]. Therefore, it is of great interest to compare these two complex structures (Figure 3). The Ssa1 C-terminal residues G⁶³⁴PTVEEVD⁶⁴¹ form an anti-parallel β -strand with the Sis1

minor β -sheet within domain I. The Ydj1-peptide substrate GWLYEIS binds Ydj1 in a similar manner. In the Sis1–Ssa1 complex structure, the hydrophobic peptide-binding depression located on domain I of Sis1 is occupied by the side chains of Val⁶³⁷ and Pro⁶³⁵ of the Ssa1 C-terminal residues G⁶³⁴PTVEEVD⁶⁴¹. In the crystal structure of Ydj1 complexed with the peptide substrate, the leucine residue in the middle of the Ydj1 peptide substrate GWLYEIS interacts with a hydrophobic peptide-binding pocket located on Ydj1 domain I [15]. However, significant differences exist between the two complexes. The Hsp70 Ssa1 C-terminal binds type II Hsp40 Sis1 primarily through charge-charge interactions and the complex formation is sensitive to high salt concentrations. Ydj1, on the other hand, binds to its peptide substrate mainly by use of hydrophobic interactions. The reduction of the hydrophobicity of the peptide-binding pocket may diminish the interactions between Ydj1 and its peptide substrate [16]. The complex formation of Ydj1 and the peptide substrate is not sensitive to salt concentrations (results not shown). The surface potential drawing of the Sis1–Ssa1 complex shows that the positively charged regions formed by the Lys¹⁹⁹, Lys²⁰², Lys²¹⁴ and Lys²⁵⁶ on domain I of Sis1 can form salt bridges with the EEVD motif within the C-terminal of Ssa1 (Figure 3A). On the surface of Ydj1, only two positively charged residues, Arg¹³¹ and Arg²¹³, could be recognized that may be responsible for the interaction with the EEVD motif of Hsp70s. The Arg¹³¹ and Arg²¹³ of Ydj1 make a less positively charged region in the Ydj1 surface compared with that of the surface of Sis1 (Figure 3B).

Sequence conservation of the interaction regions of Sis1 and Ssa1

Eukaryotic organisms, from yeast to human, encode type II Hsp40 and Hsp70 family members [7]. The crystal structure of yeast type II Hsp40 Sis1 complexed with yeast Hsp70 Ssa1 reveals that the interactions between Sis1 and Ssa1 involve both charge-charge and hydrophobic interactions. Sequence alignment of the peptide-binding fragment of eukaryotic type II Hsp40 members indicates that the positively charged residues Lys¹⁹⁹, Lys²⁰² and Lys²⁵⁶ in Sis1 that are involved in interacting with

Figure 3 Comparison of the Sis1-Ssa1 complex with the Ydj1-peptide-substrate complex

(A) The surface potential drawing of the Sis1 in the Sis1-Ssa1 complex structure determined by GRASP [26]. Blue and red denote positively and negatively charged regions respectively. The scale bar of the surface potential is shown at the top of the Figure. The bound Ssa1 C-terminal is shown in rod model. The residues of the Ssa1 C-terminal are labelled. The lysine residues of Sis1 involved in binding to the EEVD motif of the Ssa1 C-terminal are labelled. (B) GRASP presentations of Ydj1 and the peptide substrate complex structure. The residues of the peptide substrate GWLYEIS are labelled. The bound peptide GWLYEIS is shown in rod model. The residues Arg¹³¹ and Arg²¹³ of Ydj1 that may be involved in binding to the Hsp70 EEVD motif are labelled. The scale of the surface potential in (B) is very close to that in (A) as shown by the scale bar.

the Ssa1 C-terminal EEVD motif are conserved among all the family members. Lys²¹⁴ is conserved in all type I Hsp40 family members except *Drosophila* (Figure 4). The sequence alignment revealed that the hydrophobic residues forming the peptide-binding depression located on domain I of Sis1 are also conserved [13]. Alignment of the eukaryotic Hsp70 showed that the EEVD motif within the C-terminal of yeast Hsp70 Ssa1 is conserved among the family members [4]. The hydrophobicity of Pro⁶³⁵ and Val⁶³⁷ of the Ssa1 C-terminal are also conserved [4]. Therefore, the residues involved in the interactions between Sis1 and Ssa1 are

Figure 4 Sequence alignment of the C-terminal regions from eukaryotic Type II Hsp40 family members

Pileup program from the GCG package was utilized to align residues 180–352 of Sis1 from *S. cerevisiae* with similar regions of Hsp40 proteins from *Homo sapiens* (Hdj-1), *Mus musculus* (Hsp40-3), *Caenorhabditis elegans* (Z66513.1), *Drosophila Melanogaster* (Droj-1) and *Schizosaccharomyces pombe* (ψ protein). The amino acid residues of Sis1 are numbered below the alignment. The conserved lysine residues involved in binding to the EEVD motif within the family are marked by grey bars. The secondary structures of Sis1 are shown on top of the alignment. The structural components in domain I are denoted by black and those in domain II are denoted by grey. The α -helices are represented by boxes and β -strands are represented by arrows.

conserved among type II Hsp40 and Hsp70 family members. It is likely that other eukaryotic type II Hsp40 members may interact with their Hsp70 partners utilizing a similar mechanism as that for yeast Sis1 and Ssa1.

Rearrangements between domain I and II for Sis1 after Ssa1 binding

The crystal structure of Sis1 peptide-binding fragment has been determined [13]. Comparison of the Sis1 structure with the Sis1 structure within the Sis1-Ssa1 complex reveals that little conformational change occurs within the individual domains I and II of Sis1 after binding to Ssa1. However, significant domain rearrangements between domain I and domain II take place after Sis1 binds Ssa1 (Figure 5). When domain II of Sis1 is superimposed with that of the Sis1 structure within the Sis1-Ssa1 complex, we found that domain I of Sis1 in the complex structure was kinked approx 8.5° away from that in the Sis1 structure (Figure 5). These domain rearrangements were achieved by the rotation of the linker region between domain I and domain II within Sis1 (residues 258–260).

The domain rearrangements between domain I and II of Sis1 generated by the binding of the Ssa1 C-terminal swings the two I domains within the Sis1 dimer further away from each other (Figure 5). The relative positions of the II domains within the Sis1 dimer, however, are kept almost the same before and after Ssa1 binding (Figure 5). These conformational changes widen the cleft between the two Sis1 monomers within the homodimer,

Figure 5 Conformational changes of Sis1 after binding to Ssa1

(A) The Sis1 monomer structure is superimposed with the Sis1-Ssa1 complex structure after the domain II of Sis1 is aligned with that in the Sis1-Ssa1 complex. The Sis1 structure is in blue. The colour definition and molecular orientation of the Sis1-Ssa1 complex structure is the same as in Figure 1(A). (B) A view of Sis1 and the Sis1-Ssa1 complex after they have been rotated 90° along the vertical axis from the orientation shown in (A). In this Figure, only the Sis1-Ssa1 monomer is shown for clarity.

where the docking for Ssa1 peptide-binding domain may occur [6,13].

The charge-charge interactions between Sis1 and Ssa1 are important for the complex formation

The crystal structure of the Sis1-Ssa1 complex indicates that the charge-charge interactions play a significant role in complex formation. They are particularly important for Hsp40 Sis1 to bind the conserved EEVD motif of Hsp70 Ssa1. To test whether the charge-charge interactions play an important role in the Hsp40-Hsp70 complex formation, we performed structure-based mutagenesis studies. The complex crystal structure predicts that the residues Lys¹⁹⁹, Lys²⁰² and Lys²¹⁴ of Sis1 are involved in forming salt bridges with the Ssa1 EEVD motif. We constructed the mis-sense Sis1 mutants K199N, K202N and K214N to alter the positively charged properties on the Sis1 molecular surface.

The direct interactions between Sis1 and Ssa1 lid domain can be measured by use of ITC [6]. The wild-type Sis1 binds Ssa1 lid domain with the dissociation constant K_d of approx. 15 μ M (Figure 6). However, ITC cannot detect the interactions between Ssa1 lid domain and the Sis1 mutants K199N, K202N and K214N. The sensitivity of the ITC equipment allows us to detect the interactions with a K_d of approx. 200 μ M. Therefore the Sis1 mutants K199N, K202N and K214N exhibit greatly reduced binding affinities for the Ssa1 lid domain, indicating that the charge-charge interactions between Sis1 and Ssa1 play a significant role in complex formation (Figure 6).

DISCUSSION

In the present report, we show the crystal structure of the complex between the yeast Hsp40 Sis1 peptide-binding fragment and the C-terminal of Hsp70 Ssa1. The Ssa1 C-terminal residues, G⁶³⁴PTVEEVD⁶⁴¹, form a β -strand with domain I of Sis1. The

charge-charge interactions between the Ssa1 C-terminus EEVD motif and several conserved positively charged residues from Sis1 contribute significantly to complex formation. The complex formation between Sis1 and Ssa1 generates conformational changes in Sis1 to kink the individual I domains further away from each other within the Sis1 dimer.

The crystal structure of the Sis1-Ssa1 complex revealed that only the Ssa1 C-terminal residues G⁶³⁴PTVEEVD⁶⁴¹ were involved in binding to the Sis1 peptide-binding fragment in the electron density map. The majority of the Ssa1 is missing in the crystal structure probably due to its flexibility. It has been reported that the seven Ssa1 C-terminal amino acids are responsible for binding to Sis1 [6]. An 'anchoring and docking' model for Hsp70 Ssa1 interactions with Hsp40 Sis1 has been proposed, in which the Ssa1 C-terminal may function as the anchor to facilitate binding to Sis1. Our crystal structure data are consistent with these observations. A glycine-rich linker (approx. 20 amino acids in length) is present in front of the anchor region in the primary sequence of Ssa1, which may provide the flexibility that allows the efficient non-native polypeptide transfer from Hsp40 to Hsp70. The flexibility between the Ssa1 main body and its C-terminal anchor region may account for the absence of the Ssa1 main body in the crystal structure.

It is surprising to see that the yeast Hsp70 C-terminal binds Hsp40 Sis1 at the peptide-binding site, which is where Sis1 interacts with non-native polypeptides. In the protein refolding process, Hsp40 Sis1 may recognize and bind non-native polypeptides through the hydrophobic peptide-binding pocket and stretch the polypeptides into extended conformations (Figure 7). The Hsp70 Ssa1 is then recruited into this scenario. The Ssa1 C-terminal anchor region may bind the Sis1 peptide-binding pocket and replace hydrophobic side chains from the non-native polypeptide. The released hydrophobic side-chains from the non-native polypeptide may be recognized and bound by the peptide-binding groove of Hsp70 Ssa1. The non-native polypeptide swapping from Hsp40 to Hsp70 may ensure efficient refolding

Figure 6 Wild-type and mutant Sis1 interactions with Ssa1 lid domain using ITC

The panels show the heat release data after injecting the Ssa1 lid domain into the buffer containing wild-type or mutant Sis1. (A) Sis1 wild-type. (B) Sis1^{K199N}. (C) Sis1^{K202N}. (D) Sis1^{K214N}. The heat releases (vertical axis) are set at the same scale for all panels.

Figure 7 Schematic representation of the 'anchoring and docking' mechanism by which Sis1 (top) delivers a non-native peptide (black line) to Hsp70 (bottom)

The cartoon drawing depicts an Hsp70 molecule that is divided into its ATPase domain, peptide-binding groove and the lid domain. The J-domain and peptide-binding fragment of Sis1 are shown schematically. The C-terminal anchor region of Hsp70 is shown by an arrow. The black line denotes the extended non-native polypeptide. The thick black region in the non-native polypeptide indicates the hydrophobic region that can be recognized by Hsp40 and Hsp70 peptide-binding domain.

of the non-native polypeptide (Figure 7). The binding affinity between Hsp40 Sis1 and Hsp70 Ssa1 is relatively low (K_d of approx. $15 \mu\text{M}$), which ensures the dissociation of Hsp40 and Hsp70 after the non-native polypeptide transfer.

The complex formation of Sis1 and Ssa1 generate domain rearrangements between domain I and domain II in Sis1 that widen the cleft between the Sis1 monomers within the homodimer. This indicates that domain I may possess some flexibility to

take different conformations for binding to non-native polypeptides and Hsp70. The flexibility of Sis1 could be important for the Hsp40–Hsp70 system to function. The mutations within the interface of domain I and domain II in Sis1 may abolish the binding capacity of Sis1 to Ssa1, possibly because the conformational changes in Sis1 cannot be carried out in these mutations [18]. In the Sis1 structure, the peptide-binding site is occupied by a proline residue from a neighbour molecule. It is possible that the Sis1 structure may represent the conformation for Sis1 to bind the non-native polypeptide. The Sis1–Ssa1 complex structure may represent a conformation for Sis1 to interact with Hsp70 Ssa1. Hsp40 may have different conformations in these two binding states. It is not clear what roles that the Sis1 conformational changes play in Hsp40–Hsp70 molecular chaperone functions. Several possibilities exist. (1) The enlarged cleft within the Hsp40 Sis1 homodimer may provide more space for Hsp70 Ssa1 to interact with the non-native polypeptides. (2) The conformational changes may facilitate the opening of the Hsp70 peptide-binding groove by lifting the lid domain of Hsp70. (3) The conformational changes of Sis1 may help to locate the J-domain of Sis1 into a favourable position to stimulate the ATPase activity of Hsp70 Ssa1.

Type I and type II Hsp40s play distinct roles in cell physiology and cell biology [1,6–9]. The crystal structure of the Sis1–Ssa1 complex reported here, reveals that several conserved lysine residues on the surface of type II Hsp40 Sis1 are directly involved in the binding to the EEVD motif of Hsp70 Ssa1. These lysine residues make extensive positively charged regions on the Sis1 surface. However, only two arginine residues can be identified on type I Hsp40 Ydj1 surface. These two arginine residues make a less positively charged region than that on Sis1. We failed to reconstitute a stable complex of Ydj1 with the Ssa1 lid domain, whereas we did obtain the protein complex of Sis1 and the Ssa1 lid domain. These results suggest that the interactions between type I Hsp40 with Hsp70 might be different from that between type II Hsp40 with Hsp70. This could be one of the major reasons that type I and type II Hsp40s carry out distinct functions *in vivo*.

The conserved EEVD motifs in the C-terminal of Hsp70s have been shown to play critical regulatory roles for Hsp40/Hsp70 molecular chaperone functions [4]. Our crystal structure indicated that the EEVD motif of Hsp70 is directly involved in Hsp40–Hsp70 interactions by charge–charge interactions. The EEVD motif of Hsp70 can also be recognized by TPR (tetratricopeptide) domains of HOP (Hsp70 and Hsp90 organizing protein) by charge–charge interactions [24]. HOP acts as a mediator between Hsp70 and another important molecular chaperone, Hsp90 [24]. Therefore the EEVD motif of Hsp70 may play multiple roles in networking Hsp70 with other molecular chaperone members.

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