Skp is a multivalent chaperone of outer-membrane proteins

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The trimeric chaperone Skp sequesters outer-membrane proteins (OMPs) within a hydrophobic cage, thereby preventing their aggregation during transport across the periplasm in Gram-negative bacteria. Here, we studied the interaction between Escherichia coli Skp and five OMPs of varying size. Investigations of the kinetics of OMP folding revealed that higher Skp/OMP ratios are required to prevent the folding of 16-stranded OMPs compared with their 8-stranded counterparts. Ion mobility spectrometry–mass spectrometry (IMS–MS) data, computer modeling and molecular dynamics simulations provided evidence that 10- to 16-stranded OMPs are encapsulated within an expanded Skp substrate cage. For OMPs that cannot be fully accommodated in the expanded cavity, sequestration is achieved by binding of an additional Skp trimer. The results suggest a new mechanism for Skp chaperone activity involving the coordination of multiple copies of Skp in protecting a single substrate from aggregation.

β-barrel OMPs perform numerous and diverse essential functions in the outer membrane (OM) of Gram-negative bacteria. After their synthesis in the cytoplasm, OMPs are translocated across the inner membrane and then must traverse the periplasm before reaching the OM, where the β-barrel assembly machinery (BAM) complex folds them and inserts them into the membrane1–5. Whereas the periplasmic chaperones Skp and SurA are considered to be the major OMP chaperones in E. coli, a network of folding factors is involved in OMP assembly, including trigger factor and SecB in the cytoplasm, and FkpA and DegP in the periplasm1–6. Periplasmic chaperones act without an external energy source (unlike many of the Hsp chaperones8) because the periplasm is devoid of ATP5, and chaperones act without an external energy source (unlike many of the Hsp chaperones8) because the periplasm is devoid of ATP5, and they bind and release their substrates through mechanisms that are not well understood7.

The holdase chaperone Skp protects OMPs against misfolding and aggregation during their transit between the inner and outer membranes9–11. Skp has broad substrate specificity12, and its reported affinities for its substrates are in the low-nanomolar range13,14. Skp functions as a homotrimer (referred to herein as Skp), which has a ‘jellyfish’-like architecture10,11 (Fig. 1a) consisting of three α-helical ‘legs’ that extend 60 Å away from the ‘body’ domain, a nine-stranded β-barrel that mediates trimerization10,11. The three subunits of Skp form a hydrophobic cavity inside which OMP clients are bound9,12,15,16. Previous studies have suggested a 1:1 stoichiometry for all Skp–OMP complexes13,17,18. These 1:1 stoichiometries have been proposed on the basis of tryptophan fluorescence (Skp complexes formed with tOmpA (19 kDa)13, NaLP (32 kDa)13, OmpG (33 kDa)13, OmpA (35 kDa)13,17 or BamA (89 kDa)13); NMR (OmpX (16 kDa) and tOmpA (19 kDa)); and fluorescence correlation spectroscopy (OmpC (38 kDa)18). The Skp hydrophobic cavity has been estimated to be able to accommodate folded proteins of ~25 kDa (ref. 10), but many OMPs known to interact with Skp are considerably larger (for example, the 22-stranded BtuB and the 26-stranded LptD are 66 kDa and 87 kDa, respectively)12. These findings raise fundamental questions about the structural alterations that must occur to allow Skp to accommodate its larger substrates.

To investigate the mechanism by which Skp sequesters OMPs of different sizes, we used kinetic studies of OMP folding as well as ESI–IMS–MS analyses to examine the interactions of Skp with five diverse OMPs: (i) tOmpA, the eight-stranded transmembrane domain of OmpA13; (ii) PagP, an eight-stranded acyltransferase enzyme20; (iii) OmpT, a ten-stranded protease21; (iv) OmpF, a 16-stranded trimeric porin22; and (v) tBamA, the 16-stranded transmembrane domain of the BamA OMP insertase23 (Fig. 1b–f and Supplementary Table 1). We found that the concentration of Skp required to prevent OMP insertion into 1,2-diundecanoyl-sn-glycero-3-phosphocholine (dIC11,αPC) liposomes increased as the mass of client OMP increased, thus suggesting that the sequestration mechanism of Skp is altered for larger OMP clients. We used ESI–IMS–MS and computer modeling to examine the conformations of Skp–OMP complexes and provide evidence that the core of Skp expands to accommodate the larger OMPs, such that the largest clients require the formation of 2:1 Skp–OMP assemblies to completely sequester the polypeptide chain. Together, the results provide a new understanding of how Skp is able to bind, chaperone and release substrates that vary dramatically in size. These demands are met by expansion of the binding cage and/or formation of multivalent complexes.

RESULTS

Different Skp/OMP ratios are required to inhibit OMP folding

To assess the effects of Skp on the folding and membrane insertion of OMPs of varied size, we performed folding assays by dilution of

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unfolded protein stock solutions (in 8 M urea) 33-fold into buffer containing ~100-nm-diameter synthetic diC11:0PC liposomes. We then measured the increase in tryptophan fluorescence associated with folding as a function of time. Maintaining a low protein concentration (0.4 μM) and a high lipid/protein ratio (3,200:1) enables folding to be monitored in a low final concentration of urea (0.24 M) without interference from aggregation. This approach enables real-time measurements of folding that are complementary to the SDS–PAGE-based studies that have been used to provide information about the fraction of folded (SDS-resistant) protein present at a particular time24–26.

To verify that the OMPs selected for study were able to fold into diC11:0PC liposomes and/or interact with Skp under the experimental conditions used, we measured the fluorescence emission spectra of each OMP in 8 M or 0.24 M urea in the absence of liposomes (in the latter case with or without a two-fold molar excess of Skp). We then compared these spectra with those of membrane-inserted (folded) OMP obtained at the endpoint of the folding reaction (Fig. 2a–d). The spectra of tOmpA, PagP, OmpF and tBamA folded into liposomes, as compared with the spectra of the unfolded proteins in 8 M urea, showed a characteristic blueshift in maximum wavelength (λmax) and an increase in fluorescence intensity, thus indicating that a substantial fraction of all four OMPs folded successfully into the liposomes used. In the presence of Skp, the spectra of tOmpA, OmpF and tBamA showed decreases in λmax and/or intensity, as compared with the spectra of these OMPs in buffer alone, demonstrating that these unfolded OMPs interacted with Skp. For PagP, we observed no change in fluorescence in the presence of Skp, although these proteins do interact, as has been shown in previous work27.

We next investigated the effect of Skp on OMP folding kinetics. In the absence of Skp, the OMPs folded with either single- or double-exponential kinetics (Fig. 2a–d), thereby allowing the folding rate constants (k₁ and k₂) to be extracted (Fig. 2a–d and Supplementary Table 2). To verify that the transients obtained reflected membrane insertion and folding, we performed assays in the absence of lipids (Supplementary Fig. 1). A folding transient was evident only for tOmpA, PagP, OmpF and tBamA (10- and 16-stranded OMPs), bind up to two copies of Skp. The results suggested that complete sequestration of larger OMP barrels requires the binding of more than one copy of Skp.

Stoichiometries of Skp–OMP assemblies studied by ESI–MS To gain insights into the architectures of Skp–OMP assemblies, we used ESI–MS coupled with IMS to analyze different Skp–OMP complexes within multicomponent mixtures. We prepared Skp assemblies with tOmpA, PagP, OmpT, OmpF and tBamA and acquired mass spectra under instrumental conditions allowing noncovalent interactions to be retained in vacuo (termed noncovalent or ‘native’) MS28–30 (Online Methods) (Fig. 3, Supplementary Fig. 3a–f and Supplementary Table 3). The results showed that all five Skp–OMP assemblies were sufficiently stable to survive the ESI process and to be transferred into the gas phase for analysis.

Several reports have suggested that Skp binds unfolded OMPs ranging from 16 to 89 kDa (8–16 β-strands in the native state) with a 1:1 stoichiometry13,15,17,18. By contrast, the mass spectra (Fig. 3) revealed that the stoichiometry of these assemblies is dependent on the size of the OMP client, a result consistent with the kinetic traces described above. Thus, tOmpA and PagP (eight-stranded OMPs) bind only one Skp, whereas the larger OMPs, OmpT, OmpF and tBamA (10- and 16-stranded OMPs), bind up to two copies of Skp. We also observed peaks corresponding to monomeric Skp subunits.
(m/z ~2,000) (Fig. 3), results indicating either that some dissociation of the assembly occurred in-source and/or reflects the population of monomeric subunits in solution\(^{31,32}\). Interestingly, a 2:1 assembly was the predominant complex observed in the spectrum for the largest 16-stranded OMP studied, tBamA, although the complex was formed by mixture of Skp with tBamA at a 1:1 molar ratio (Fig. 3f). We also observed a 2:1 Skp–OMP assembly for full-length BamA (Supplementary Fig. 4). To confirm that the Skp/OMP stoichiometry observed through ESI–MS reflected the stoichiometry in solution, we performed chemical cross-linking with bis(sulfosuccinimidyl)suberate (BS3) and subsequent SDS–PAGE analysis of Skp preincubated with full-length OmpA or full-length BamA. In the cross-linked Skp–OmpA samples, we observed a 1:1 assembly (Supplementary Fig. 5a–d), whereas in the Skp–BamA samples, we observed a band consistent with a complex with a 2:1 stoichiometry (but no 1:1 Skp–BamA complex) (Supplementary Fig. 5e–g).

**Insights into Skp–OMP-complex structure from ESI–IMS–MS**

We next used ESI–IMS–MS to analyze how Skp binds its OMP clients of larger size. IMS measures the mobility of ions through an inert-gas-filled chamber under the influence of a weak electric field, and the drift time (mobility) of an ion in this environment is dependent on the ion’s mass, size and charge\(^{28,33}\). Here, we used traveling wave IMS–MS\(^{28,33}\), for which calibration of the measured drift-time data can be performed to obtain rotationally averaged collision cross-sections (CCSs) and consequently gain insight into the conformations of Skp and the Skp–OMP assemblies. We acquired IMS data for all of the assemblies studied (Fig. 4a–f and Supplementary Fig. 3a–f) and compared them with known structures, if available, or with models of the Skp–OMP complexes for which there are no high-resolution structural data.

We determined the CCS distributions of the observed ions originating from Skp and 1:1 Skp–OMP complexes, normalized to spectral intensity (Fig. 4a–f), and we further plotted the modal CCSs as a function of charge state (Fig. 4g and Supplementary Table 4). Interestingly, the CCSs of the Skp ions (Fig. 4g) were smaller than expected on the basis of the published Skp crystal structure (in which the modal CCSs at the lowest observed charge state, which is least affected by Coulombic repulsion\(^{34}\), was 37.9 nm\(^2\), approximately 25% lower than the expected value of 45.7 nm\(^2\) derived from the crystal structure (Fig. 4g)). Molecular dynamics (MD) simulations (Supplementary Fig. 6 and Supplementary Data Sets 1 and 2) revealed that the assembly collapses in the gas phase, thus resulting in a structure with a CCS of 37.3 ± 1.9 nm\(^2\) (mean ± s.d. of three MD simulations) (Fig. 4g).

Binding of Skp to the eight-stranded tOmpA and PagP (Fig. 4g), compared with Skp alone, resulted in ions with increased CCSs. The CCSs of the ions observed (45.6 nm\(^2\) and 45.8 nm\(^2\) for Skp–tOmpA and Skp–PagP, respectively, at the lowest observed charge state) were...
Figure 3  Skp–OMP complexes have different stoichiometries. (a–f) ESI mass spectra of Skp (5 μM) (a) and Skp preincubated in the presence of 5 μM tOmpA (b), PagP (c), OmpT (d), OmpF (e) and tBamA (f). The most abundant charge state is labeled for each distribution. Observed masses of the complexes are summarized in Supplementary Table 3. Complementary IMS data are shown in Supplementary Figure 3. Source data for mass spectra are available online.

Comparing the CCSs predicted from the crystal structure of Skp alone (45.7 nm²), thus supporting the notion that these assemblies are specific complexes in which the OMP is located within the central Skp cavity and prevents collapse of the chaperone in the gas phase15,16. Consistently with this possibility, the Skp–OMP complexes sampled a narrower conformational ensemble than did Skp alone, as measured by the width at half height of the mobility peaks observed (Fig. 4a–d). Complexation with tOmpA and PagP thus packs the hydrophobic cavity of Skp, resulting in a narrower conformational ensemble consistent with previous data15.

Interestingly, the 1:1 Skp–OMP assemblies of the larger OMPs studied (OmpT, OmpF and tBamA) (Fig. 4g) had larger CCSs (51.4–54.2 nm²) than those of the Skp–tOmpA and Skp–PagP assemblies (~46 nm²). These data, together with the mismatch between the volume of Skp's cavity and the volume likely to be occupied by larger OMP clients in the 'fluid globule' state15, suggest that the central cavity expands in size to allow encapsulation of these species, in agreement with recent SANS data35. However, our kinetic data (Fig. 2c,d) indicated that Skp expansion is insufficient to fully sequester these larger OMPs in a 1:1 complex.

Plotting the increase in CCS as a function of molecular weight for Skp and all complexes (Fig. 4h), including 2:1 Skp–OMP assemblies (Supplementary Fig. 3g), revealed that the data fit to a globular model, irrespective of client size. The complexes exhibited an effective gas phase density of 0.33 Da Å⁻³, a value both similar to those reported for other protein complexes33 and consistent with recent calculations of the CCSs of globular proteins in the Protein Data Bank36.

Modeling of larger OMPs in complex with two copies of Skp

We next generated models of the architecture of Skp in complex with OmpT, tBamA or OmpF to determine how Skp and a partially folded
Figure 5 Possible architectures of Skp–OMP complexes. (a-f) Side-view (left) and bottomview (right) surface representations. Models of Skp are based on the published crystal structure (PDB 1U2M10), with missing residues modeled by molecular replacement. (a) Skp (yellow) bound to an eight-stranded OMP represented by a gray sphere of radius 20 Å. (b) Skp (yellow) with an expanded central cavity surrounding a 16-stranded OMP represented by a gray sphere of radius 25 Å. (c,d) 2:1 Skp–OMP structures (with Skp in yellow and green) arranged side by side in a parallel (c) or antiparallel (d) arrangement, with the 16-stranded OMP substrate represented by a gray capsule with a cylinder height of 37 Å and cap radii of 20 Å. (e) 2:1 Skp–OMP complex with an interlocked architecture, with the 16-stranded OMP represented by a gray sphere of radius 25 Å. (f) The hexameric eukaryotic chaperone prefoldin (PDB 1FXK37), with chains A, B and C of the crystal structure shown in green, cyan and yellow, respectively.

OMPs might interact. We constructed four different models. As a starting point, we modeled encapsulated tOmpA as a sphere with a radius of 20 Å (Fig. 5a) and a volume of 33,500 Å³, values consistent with previous estimates10,15. We assumed that the amino acid density for non-native OMPs bound to Skp is independent of the mass of the OMP studied, in agreement with the MS data presented above. Therefore, to model a 16-stranded OMP, we assumed a spherical volume of ~67,000 Å³ and hence a radius of ~25 Å, and generated a Skp model with its three subunits surrounding a sphere of this size (Fig. 5b). The theoretical CCS of the resulting structure (50.4 nm²) (Fig. 4g) was in good agreement with the measured CCS values for 1:1 Skp–tOmpT, Skp–tOmpF and Skp–tBamA complexes (51.4, 51.8 and 54.2 nm², respectively) (Fig. 4g and Supplementary Table 4). The results therefore suggest that 1:1 Skp–OMP complexes with larger OMPs involve an expanded Skp cavity.

Next, we generated models of the 2:1 Skp–OMP complexes, theorizing that Skp might arrange in a side-by-side configuration, in either a parallel or antiparallel arrangement (Fig. 5c,d), with the OMP represented by a capsule. We determined the theoretical CCSs of these assemblies to be 79.1 and 78.2 nm², respectively (Fig. 4g). Alternatively, we considered a model in which the OMP substrate (represented by a sphere with a radius of ~25 Å) might be encapsulated by two interlocking copies of Skp (Fig. 5e), thus resulting in a complex with a theoretical CCS of 73.5 nm² (Fig. 4g). All three values were in good agreement with the measured CCS values for 2:1 Skp–tOmpT, Skp–tOmpF and Skp–tBamA complexes (71.7, 71.2 and 72.8 nm², respectively) (Fig. 4g and Supplementary Table 4).

Molecular dynamics simulations to model Skp–OMP complexes
To model the Skp–OMP complexes further and aid in their visualization, we performed a series of MD simulations. A simulation of apo-Skp in explicit solvent over 100 ns demonstrated that the individual subunits are highly dynamic and flexible. Each subunit undergoes a transition to an ‘open’ state, in which subunit helices splay from the central axis, thereby resulting in an expanded central cavity consistent with the results of previous MD studies16,35 (Supplementary Fig. 7a–c, Supplementary Data Sets 1 and 3 and Supplementary Video 1). The average radius of gyration (Rg) of Skp from the simulation (31.5 Å) was in good agreement with published SANS data (~33 Å)35. This Rg was ~10% higher than that predicted from the crystal structure, indicating that dynamic motions of the Skp subunits observed in the simulation probably reflect those in solution. In the two Skp crystal structures solved to date10,11, the lower section of one of the subunits is unresolved, thus indicating flexibility, and the angles with which the Skp subunits extend away from the body domain are different for each of the subunits in the two structures10,11. The subunits in the crystal structure of the heterohexameric eukaryotic chaperone prefoldin (Fig. 5f) also form different angles with respect to the multimerization domain, which has also been suggested to indicate conformational flexibility that may be functionally relevant37.

Next, we generated models of (8-stranded) tOmpA and (16-stranded) tBamA alone in an unfolded, extended conformation and simulated their behavior in solvent (mimicking the situation in which OMPs are diluted from 8 M urea). In each case, the OMPs collapse rapidly into an approximately globular form (Supplementary Fig. 7d,e and Supplementary Video 2). We then generated a model of the 1:1 Skp–tOmpA complex by placing the collapsed tOmpA structure within the cavity of Skp in an open conformation from

Figure 6 In vacuo MD simulations of 1:1 and 2:1 Skp–OMP complexes. (a,b) Starting model of a 1:1 Skp–tOmpA complex used for MD simulations (Supplementary Data Set 4) (a) and the structure obtained after 10 ns of in vacuo simulation (Supplementary Data Set 5) (b). (c,d) Starting model of a 2:1 Skp–tBamA complex used for MD simulations, with the two copies of Skp arranged in a side-by-side parallel orientation (Fig. 5c) (Supplementary Data Set 6) (c) and the structure obtained after 10 ns of in vacuo simulation (Supplementary Data Set 7) (d). Views from the side (top) and bottom (bottom) are shown. Skp (green and blue) is shown in cartoon representation. OMPs (yellow) are shown in surface representation. Representative structures from three independent MD simulations are shown.
the simulation of apo-Skp (Supplementary Fig. 7b) and relaxing the resulting structure in vacuo. In the simulation, the subunits of Skp collapse rapidly around the tOmpA substrate, thus resulting in a structure with a CCS value of $43.7 \pm 1.2 \text{nm}^2$. In excellent agreement with that measured by ESI–IMS–MS ($45.6 \pm 0.1 \text{nm}^2$), these results suggest that, at least in the gas phase, Skp 'clamps' around the substrate (Fig. 6a,b and Supplementary Video 3). We created a model for the gas-phase 2:1 Skp–tBamA complex by placing the collapsed tBamA structure in the hydrophobic cavity formed by two copies of Skp in their open conformations. We chose a side-by-side parallel orientation (Fig. 6c) on the basis of the striking resemblance of this model to the structure of the eukaryotic prefoldin chaperone29 (Fig. 5f). The size of the collapsed tBamA model clearly exceeds the maximal dimensions of the cavity of a single Skp observed in an open conformation (Fig. 6c). Simulation of the 2:1 Skp–tBamA complex in vacuo showed that the Skp subunits also rapidly clamp around the tBamA substrate, thus creating a complex with a CCS of $74.4 \pm 1.4 \text{nm}^2$, a value again in good agreement with the IMS data (72.8 $\pm 0.2 \text{nm}^2$) (Fig. 6d and Supplementary Video 4). Thus, the CCS data obtained from both experiment and simulation (Supplementary Table 5) are consistent with a model in which binding of multivalent Skp is necessary to sequester OMPs that exceed the dimensions of the Skp cavity.

To provide evidence that a similar clamping motion of Skp around its OMP substrates could occur in solution, we performed analogous MD simulations of Skp–tOmpA and 2:1 Skp–tBamA complexes in explicit solvent. In these simulations, Skp subunits also wrap around their OMP substrates (Fig. 7a,b and Supplementary Videos 5 and 6), with clamping movements similar to those observed in the gas-phase simulations (Fig. 6). The complexes formed are stable over 100 ns (Fig. 7c,d) and have larger calculated CCS values ($56.5 \pm 0.3 \text{nm}^2$ and $101.2 \pm 6.0 \text{nm}^2$ for Skp–tOmpA and 2:1 Skp–tBamA, respectively) than those after gas-phase simulation ($43.7 \pm 1.2 \text{nm}^2$ and $74.4 \pm 1.4 \text{nm}^2$ for Skp–tOmpA and 2:1 Skp–tBamA, respectively). These data are consistent with a model in which the subunits of Skp are dynamic and result in expansion of the hydrophobic cavity, thereby allowing entry of substrates of varying sizes, before the Skp subunits wrap around the sequestered client and protect it from aggregation until client folding into the bilayer can take place.

**DISCUSSION**

Major advances in the understanding of the cascade of molecular chaperones and folding catalysts involved in OMP biogenesis have been made in recent years, yet the molecular details of how OMPs are bound by molecular chaperones, transported across the periplasm and assembled into the outer membrane, without using the energy of ATP binding and hydrolysis, remain unclear.1,2,23, Here, we provided new insights into how Skp is able to chaperone its broad array of OMP clients, including substrates that are too large to be accommodated within its hydrophobic cavity. We demonstrated that Skp uses subunit dynamics to expand the size of its client-binding cavity and that Skp functions as a multivalent chaperone that sequesters and prevents aggregation of its larger OMP clients. Further, we used ESI–IMS–MS to gain structural insight into the 1:1 and 2:1 Skp–OMP complexes that we identified. Using kinetic refolding and ESI–IMS–MS data combined with MD simulations, we generated models consistent with the experimental results in which Skp sequesters larger OMPs by binding in a multivalent arrangement (side-by-side parallel or antiparallel, and/or via an interlocking structure) (Fig. 5c–e). The parallel side-by-side model (Fig. 5c) bears a striking resemblance to the structure of the nonhomologous chaperone prefoldin57 (Fig. 5f). Resolving the precise orientations of Skp molecules in these multivalent complexes will require more information, for example from cross-linking experiments followed by MS/MS. Nonetheless, our biochemical, MS and MD results indicate that the ability of Skp to chaperone OMPs ranging from 35 to 43 kDa in size requires both subunit dynamics and Skp's ability to function as a multivalent chaperone.

Skp has been shown in vivo to interact with much larger substrates than those investigated here (19–43 kDa), including BtuB (66 kDa), FhuA (79 kDa) and LptD (87 kDa) which form β–barrels composed of 22, 22 and 26 β-strands, respectively.12,38 It is likely these proteins also form multivalent complexes with Skp, and indeed recent data on the interaction between Skp and FhuA are consistent with a Skp/OMP stoichiometry greater than 1:1 (ref. 39).

The present results have implications for the understanding of how OMPs are chaperoned by Skp in the periplasm, including the mechanisms of substrate binding and release. Our atomistic MD trajectories of apo-Skp showed that it exists in a wide range of open conformations and exhibits large differences in the area of the cavity entrance formed between the tips of its three subunits. Such conformational flexibility has been implicated in the mechanisms of other ATP-independent chaperones40,41. We propose that in rescuing aggregation-prone proteins, Skp may be thought of as being analogous to a pair of ‘calipers’ that samples open conformations before client capture and consequently adjusts the volume of its central cavity. In this model, once the substrate has entered the Skp cavity, the Skp subunits clamp down and protect the exposed hydrophobic surfaces of the protein, in a mechanism similar to those of other chaperones such as trigger factor or Hsp90 (ref. 42). For substrates too large to be accommodated within the Skp substrate cavity, additional copies of Skp recognize and engulf sections of the substrate not already encapsulated. In vivo cross-linking evidence has suggested
that Skp can interact with OMPs as they emerge from the SecYEG translocon. Thus, it is possible that during translocation of larger OMPs, the substrate is fed directly into the cavity of Skp, and the chaperone's maximum binding capacity might be reached before the complete polypeptide chain is translocated. Subsequent polypeptide chain emerging from the translocon could then be bound by a second or more Skps, thereby ensuring sequestration of the entire polypeptide sequence so that periplasmic aggregation is prevented.

Recent equilibrium sedimentation experiments of Skp in the absence of substrate have demonstrated a dynamic equilibrium between folded subunit monomers and trimers at physiological concentrations. Therefore, a possible alternative in vivo pathway to the formation of Skp–OMP complexes may involve sequential binding of monomer subunits to OMP substrates, such that Skp trimerization is linked to (and indeed driven by) substrate binding.

It has been proposed that transient exposure of the C-terminal OMP-targeting sequence (β-signal), and its recognition by the BAM complex, triggers substrate release from Skp. The space between Skp subunits (~25 Å in the crystal stucture) and/or the inherent dynamics of the complex may facilitate the transient solvent exposure of regions of the OMP substrate, thus permitting β-signal recognition. Consistently with this possibility, the presence of BamA in liposomes has been found to relieve the folding inhibition of OmpA by Skp in vitro. Interestingly, all 2:1 Skp–OMP models proposed here retain a substantial distance (~20 Å) between the Skp subunits, and this distance would permit exposure of sections of the substrate polypeptide required for BAM signaling and/or membrane insertion. The release of the OMP from Skp is likely to be driven by the increased thermodynamic stability of the folded OMP relative to the chaperone-bound state, and, for OMPs bound to more than one Skp, it is possible that individual copies of Skp might be released sequentially in a process driven by the free energy of OMP folding.

Chaperones utilize two general strategies to protect substrates from misfolding and aggregation. In the first, substrates are chaperoned by sequential binding and release of exposed hydrophobic surfaces along an extended polypeptide chain. This ‘beads on a string’ model is typified by chaperones such as the Hsp70s and trigger factor. Alternatively, aberrant interactions may be prevented by sequestration of the substrate from the cellular environment within an enclosed space, as is the case for the ‘cage-like’ chaperonins such as GroEL and GroES, and TrIC. The data presented here suggest that Skp operates with a ‘hybrid’ mechanism, using both of these strategies to bind and encapsulate its OMP client, thereby preventing their aggregation and facilitating their delivery to the OM, where folding can successfully occur.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
B.S. and A.N.C. contributed equally to this work. B.S. designed and performed the kinetic experiments, computer modeling and MD simulations. A.N.C. designed and performed the MS and cross-linking experiments. P.W.A.D. designed and performed in vacuo apo–Skp simulations. S.A.H. assisted and provided supervision in the MD simulations. A.E.A., D.J.B. and S.E.R. conceived, designed and supervised the research. All authors contributed to the discussion and were involved in editing the final manuscript.

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Expression and purification of PagP. Expression and purification of PagP was carried out as previously described.27 Protein concentrations were determined spectrophotometrically by using molar extinction coefficients at 280 nm of 82,390 M⁻¹ cm⁻¹ for PagP, OmpA, OmpF, and BamA, respectively.

Expression and purification of OmpT. Expression and purification of OmpT was carried out as previously described.26 The plasmids for tOmpA, OmpT, and BamA encoding the mature OMP sequences were kindly provided by K. Fleming (John Hopkins University).25

Expression and purification of histidine-tagged Skp. Histidine-tagged Skp was expressed and purified with a protocol adapted from Burmann et al.15

Expression and purification of histidine-tagged PagP, tBamA and OmpT. A codon-optimized synthetic gene (Eurofins) of the mature sequence of OmpF was cloned into pET11a (Novagen) between the NdeI (5′) and BamHI (3′) restriction sites. To create the tBamA construct, residues 425–810 of BamA were amplified by PCR, with plasmid BamAB-pETDuet-1 (kindly donated by S. Buchanan (NIH)) as the template, and the resultant product was then ligated into pET11a as described above. The OmpT mature sequence (residues 21–217) was amplified by PCR from E. coli XL1-blue cells to include an N-terminal His, tag and TEV protease-cleavage site (MHL6ENLYFQG-OmpT), and was subsequently cloned into the pET11a vector as described above. The plasmids for tOmpA, PagP, OmpA and BamA encoding the mature OMP sequences were kindly provided by K. Fleming (John Hopkins University).25

Preparation of liposomes. 1,2-diiodoecanoyl-sn-glycerol-3-phosphocholine (diC11:0PC or DUPC) lipids were purchased from Avanti Polar Lipids. DUPC was obtained as a powder, dissolved in a 80:20 chloroform/methanol mixture at 25 mg/mL and stored at −20 °C until use. Appropriate volumes were transferred to glass test tubes, and an even lipid film was created by drying under a gentle stream of nitrogen during moderate shaking in a 42 °C water bath. Lipid films were further dried in a vacuum desiccator for >3 h and then resuspended in 50 mM glycine-NaOH, pH 9.5, to a concentration of 40 mM. Resuspended lipids were vortexed briefly and allowed to stand for 30 min. After being vortexed again, lipids were subjected to five freeze-thaw cycles with liquid nitrogen. Large unilamellar vesicles (LUVs) (100 nm) were prepared by extrusion of the lipid suspension 11 times through a 0.1-µm polycarbonate membrane (Nuclepore) with a mini extruder (Avanti Polar Lipids). Liposomes were stored at 4 °C and used within 48 h of preparation.

Kinetic folding assays. Kinetic measurements were carried out with a Quantum Master Fluorometer (Photon Technology) controlled by FelixGX software v4.3. For each experiment, four separate samples were run in a four-cell changer maintained at 25 °C by a Peltier controlled temperature unit. The tryptophan fluorescence of samples was excited at a wavelength of 295 nm, and fluorescence emission was monitored at 335 nm. 295 nm rather than 280 nm was chosen as the excitation wavelength to minimize the fluorescence-intensity contribution from Skp, which contains tyrosine but no tryptophan residues. The excitation slit widths were set to 0.4–0.6 nm, and the emission slit widths were set to 5 nm. The high emission/excitation slit-width ratio was important to minimize photo bleaching on the experimental timescale. OMPs were buffer-exchanged from 25 mM Tris−HCl and 6 M Gdn−HCl, pH 9.5, into 50 mM glycine-NaOH and 8 M urea, pH 9.5, with Zeba spin desalting columns (Thermo Scientific) and diluted to 80 µM. OMP folding reactions in the absence of Skp were initiated by manual dilution of OMPs from the 80 µM unfolded protein stock in 8 M urea to a final concentration of 0.4 µM protein and 0.24 M urea in the presence of 1.28 mM DUPC liposomes (lipid/protein molar ratio (LPR) of 3,200:1), in 50 mM glycine-NaOH, pH 9.5. Stable folding of Skp in 0.24 M urea was verified through far-UV spectroscopy (data not shown). The final volume of each sample was 500 µL. For Skp−OMP experiments, OMPs were preincubated with Skp for approximately 1 min before addition of liposomes. OMPs were diluted and mixed from an 80 µM stock in 8 M urea to a final concentration of 2.4 µM, in the presence of the appropriate molar ratio of Skp, in 0.24 M urea and 50 mM glycine-NaOH, pH 9.5 (no lipids). This Skp−OMP stock was then further diluted 6× in the presence of 1.28 mM DUPC in 0.24 M urea and 50 mM glycine-NaOH, pH 9.5, to begin the assay. The final volume of each sample was 540 µL. At the concentrations of Skp used here, Skp has been shown to be in a dynamic equilibrium between folded monomer subunits and trimers.25 All Skp concentrations referred to here are trimers equivalents. For each experiment with a particular liposome batch, four samples were measured concurrently. A minimum of three replicates were globally fitted with IgorPro 6.0 (WaveMetrics) to extract rate constants, and the fits were forced to share the same rate constants. Transients were fitted either to a single-exponential function:

$$y = A_1 \times e^{-k_1 t} + c$$

or to a double-exponential function:

$$y = (A_2 \times e^{-k_2 t}) + (A_1 \times e^{-k_1 t}) + c$$

where $k_1$ and $k_2$ are rate constants, $A_1$ and $A_2$ are their associated amplitudes, and $c$ is a constant. Transients were fitted to a double-exponential function if a satisfactory fit was not obtained to a single-exponential function, as judged by inspection of residuals. Experiments were performed for each condition with three separate liposome batches, and reported errors are the s.d. of rate constants among liposome batches.
Fluorescence emission spectra. Fluorescence emission spectra were acquired on the same instrument used for the kinetic assays (above). Each spectrum was recorded from 305 nm to 400 nm in 1-nm increments, with an excitation wavelength of 295 nm. All spectra were acquired at 25 °C, and all samples contained 50 mM glycine-NaOH, pH 9.5, in a sample volume of 500 μL. OMPs from an 80 μM stock in 8 M urea were diluted to a final concentration of 0.4 μM in the presence of a two-fold molar excess of Skp in 0.24 M urea or 8 M urea, or buffer alone in 0.24 M urea. Folded samples were prepared by dilution of an 80 μM OMP stock to 0.4 μM in the presence of 1.28 mM DUPC liposomes (molar LPR 3,200:1) in 0.24 M urea and incubated at 25 °C for ~1.5 h before acquisition of the fluorescence emission spectra.

Mass spectrometry. Skp–OMP complexes were prepared by rapid dilution of the denatured OMP (400 μM in 8 M urea and 50 mM glycine-NaOH, pH 9.5) to a final concentration of 5 μM into a solution of Skp (5 μM in 50 mM glycine-NaOH, pH 9.5). The samples were then buffer-exchanged into 200 mM ammonium acetate, pH 10, with Zeba spin desalting columns (Thermo Scientific) immediately before MS analysis. NanoESI–IMS–MS spectra were acquired with a Synapt HDMS mass spectrometer (Waters) with platinum/gold-plated borosilicate capillaries prepared in house. Typical instrument parameters were: capillary voltage, 1.2–1.6 kV; cone voltage, 40 V; trap collision voltage, 6 V; transfer collision voltage, 10 V; trap DC bias, 20 V; backing pressure, 4.5 mbar; IMS gas pressure, 0.5 mbar; traveling wave voltage, 7 V; and traveling wave velocity, 250 ms−1. Data were processed with MassLynx v4.1, Driftscope 2.5 (Waters) and MassLynx v4.1 (Waters). Estimated modal CCSs were shown as mean ± s.d. from three independent experiments. Theoretical CCSs for globular proteins with a given effective gas phase density were calculated according to previously described methods.[57]

Chemical cross-linking and SDS–PAGE analysis. Samples for chemical cross-linking were prepared by rapid dilution of urea-denatured OMP into an Skp solution (in 20 mM HEPES, pH 7.5, and 150 mM NaCl) at 4 °C, so that the final concentrations of Skp and OMPs were 20 μM in a final concentration of 0.24 M urea. Samples were mixed for 2 min and centrifuged (20 min, 13,000g, 4 °C) to remove aggregated material. The supernatant was removed, and BS3 (Thermo Scientific) was added at a 50-fold molar excess over the Skp concentration. Samples were incubated at room temperature for 30 min before the addition of 250 mM glycine, pH 9.5. Samples were analyzed through SDS–PAGE, and the gels were stained with Coomassie blue and washed with 50% acetonitrile in 25 mM ammonium bicarbonate (10 min). Gel bands were excised, cut into 1 mm pieces, destained with 30% ethanol and washed with 50% acetonitrile in 25 mM ammonium bicarbonate (10 min). The gel pieces were then dehydrated with acetonitrile (5 min), and the residual volatile solvent was removed by evaporation. The pieces were then rehydrated with a solution of trypsin (Promega) (20 ng μL−1 in 25 mM ammonium bicarbonate) and incubated at 37 °C for 18 h. Peptides were recovered by three rounds of addition, incubation and removal of 60% acetonitrile/5% formic acid to the gel pieces. The samples were then pooled and concentrated.

Peptides were analyzed through data-dependent LC–MS/MS on a nanoAcquity LC system interfaced with a Synapt G2-Si HDMS mass spectrometer (Waters). Peptides (1 μL) were injected onto an Acquity M-Class C18, 75 μm × 150 mm column (Waters) and then separated by gradient elution of 1–50% solvent B (0.1% (v/v) formic acid in acetonitrile) in solvent A (0.1% (v/v) formic acid in water) over 60 min at 0.3 μL min−1. Mass calibration was performed through a separate injection of aqueous sodium iodide at a concentration of 2 μg/μL. [Glu1]-fibrinopeptide B was infused as a lock mass calibrant. Data acquisition was achieved through data-dependent analysis with a 1-s MS scan over an m/z range of 350–2,000, and this was followed by four 0.5-s MS/MS scans of the four most intense ions in the MS spectrum. Data processing was performed with PEAKS Studio 7 (Bioinformatics Solutions).

Modeling of Skp–OMP complexes. All modeling was performed with PyMOL (http://www.pymol.org/). To generate models of the Skp–OMP complexes, the missing residues in chains B and C of the Skp crystal structure (PDB 1U2M[58]) were modeled from chain A. For the Skp–tOmpA/PagP model (Fig. 5a), the OMP was modeled as a sphere of radius 20 Å with its origin positioned at the geometric center between the α-carbon atoms of residue 50 of each Skp chain. For the Skp–OMP model with expanded Skp subunits (Fig. 5b), each chain was positioned around a sphere of radius 25 Å representing the larger OMP. The flexible tips of each subunit (residues 51–101) were modeled hinged slightly inward to wrap around the substrate. The side-by-side parallel and antiparallel 2:1 Skp–OMP models (Fig. 5c,d) were created by duplication of the Skp–tOmpA/PagP model (Fig. 5a) and appropriate rotation and translation. The interlocking-trimer 2:1 Skp–OMP model (Fig. 5e) was generated by duplication of the Skp model with an expanded cavity (Fig. 5b) and appropriate rotation and translation of the duplicated Skp. Theoretical CCS values were generated by using the calibrated trajectory method implemented in IMPACT.[59,60]

Molecular dynamics simulations. MD simulations were prepared with the AmberTools 14 suite of programs and performed with AMBER and the ff14SB force field[60]. To simulate apo-Skp in water, a Skp model was first generated from the Skp crystal structure (PDB 1U2M[58]) with the residues absent in chains B and C modeled from chain A. After addition of hydrogen atoms with Xleap, Skp was placed in a TIP3P water box with a 10.0-Å cutoff, and the system was neutralized with a total of 15 Cl− ions. The system was equilibrated through an initial energy minimization, which was followed by 80 ps of restrained MD during which the system was heated to 300 K with gradual releasing of restraints. An unrestrained MD simulation of 100 ns was then performed.

Simulations of the collapse of the extended chains of tOmpA and tBamA were carried out with a generalized Born/solvent accessible surface area (GB/SA) implicit solvent model[59,60]. Use of an implicit solvent model speeds up the exploration of conformational space by at least an order of magnitude because of the neglect of frictional forces from collisions with water molecules,[61] thereby leading to rapid adoption of a collapsed configuration from the initially linear structure (Supplementary Fig. 7d,e and Supplementary Video 2). The polypeptide starting structures were generated in Xleap and, after initial energy minimization, were simulated for 3 ns. The starting models for the simulation of Skp–tOmpA and Skp–tBamA in vacuo were created in PyMOL by positioning OMPs, after simulated collapse, within the cavity of Skp-trimer structures in an open conformation taken from the explicit solvent apo-Skp simulation. All simulations except apo-Skp in explicit water were performed in triplicate. Each in vacuo simulation of Skp–tOmpA and 2:1 Skp–tBamA was performed with a starting OMP structure from a different simulation. In vacuo simulations of apo-Skp were performed for three different starting structures selected from the simulation of apo-Skp in explicit water. For all in vacuo simulations, the system was equilibrated with an initial energy minimization, and this was followed by eight steps of restrained MD during which the system was heated to 390 K with gradual releasing of restraints. Subsequently, an unrestrained MD simulation of 100 ns was performed.

To simulate the 1:1 Skp–tOmpA and 2:1 Skp–tBamA complexes in solution, starting models were generated as detailed above for the in vacuo simulations. The Skp–OMP complexes were placed in a TIP3P water box with a 10.0-Å cutoff, and the system was neutralized with a total of 10 Cl− ions (1:1 Skp–tOmpA) or 12 Cl− ions (2:1 Skp–tBamA). The systems were equilibrated with an initial energy minimization, and this was followed by 80 ps of restrained MD during which the system was heated to 300 K with gradual releasing of restraints. Subsequently, an unrestrained MD simulation of 100 ns was performed. For each complex, simulations were repeated in triplicate.

For comparison with IMS–MS data, theoretical CCSs of final structures at the ends of simulations were calculated with the trajectory method and IMPACT.[59] CCS values for all structures after in vacuo simulations were obtained after 100 ns of unrestrained simulation. CCS values for tOmpA and tBamA in implicit solvent were obtained after 3 ns of unrestrained simulation. The integration time step was 2 fs, and atomic positions were saved every 500 steps (1 ps). VMD software was used to compute backbone r.m.s. deviations and to render videos of the simulations. Analyses of Rg changes over trajectories were carried out with ptraj.
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