Non-canonical Interactions between Heat Shock Cognate Protein 70 (Hsc70) and Bcl2-associated Anthanogene (BAG) Co-Chaperones Are Important for Client Release

Received for publication, June 7, 2016, and in revised form, July 26, 2016. Published, JBC Papers in Press, July 29, 2016, DOI 10.1074/jbc.M116.742502

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Heat shock cognate protein 70 (Hsc70, HSPA8) is a molecular chaperone that regulates the folding, trafficking, function, stability, and turnover of its client proteins (1, 2). The versatility of the Hsc70 system is thought to arise from its association with co-chaperones. Co-chaperones bind to Hsc70 and, among other activities, help shuttle clients to cellular systems dedicated to folding, turnover, and other fates (3, 4). Accordingly, there is an interest in understanding the protein-protein interactions between Hsc70 and its co-chaperones because these contacts are important for “fine tuning” protein quality control and homeostasis.

Hsc70 is a 70-kDa chaperone that is composed of a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD) (5). The SBD is further divided into a β-sandwich subdomain that contains a hydrophobic cleft for client binding (6), a helical “lid” subdomain that controls access to the cleft and a disordered C-terminal domain (CTD), which is a site of post-translational modifications and protein-protein interactions (7, 8). On the other side of the chaperone, the NBD is subdivided into two lobes (lobe I and lobe II) that each contributes to a nucleotide-binding cleft in which ATP is bound and hydrolyzed. Much of our knowledge of Hsc70 structure and dynamics has come from studies on the prokaryotic ortholog, DnaK. However, the high sequence conservation of this family of proteins (often ~50% identity) suggests that similar mechanisms might be relevant across orthologs and paralogs (9). Despite this high level of homology, there also appears to be some differences in the regulation of the prokaryotic and eukaryotic family members (10, 11), including in the way that they interact with co-chaperones (12).

One of the most striking structural features of Hsc70/DnaK is the large motions of the NBD that accompany ATP binding and conversion to the ADP-bound state (13–15). These motions are, in turn, propagated into the SBD through a short, conserved linker and an allosteric network (16–19). Briefly, binding to ATP favors an “open” configuration of the lid subdomain (20), which occurs concurrent with docking of the NBD to the SBD. This ATP-bound conformer has relatively weak affinity for clients, as the client’s off-rate is increased when the lid is open (21, 22). To continue the cycle, hydrolysis of nucleotide reorganizes lobes I and II, which “closes” the lid subdomain and strengthens the affinity for clients. In this ADP-bound state, the NBD and SBD are no longer docked; rather, they move relatively independently in solution (23). Because of these dramatic conformational changes, Hsc70/DnaK is often used as a model for intra- and inter-domain allostery in multidomain proteins (5, 15).

Each step of Hsc70’s conformational cycle is regulated by protein-protein interactions with co-chaperones. In eukaryotes, the BAG family of co-chaperones, including Bag1, Bag2, Bag3, Bag4, and Bag5, act as nucleotide-exchange factors (NEFs): promoting the release of ADP and client from the Hsc70 complex...
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(4, 24, 25). This activity is important because it resolves a potentially rate-limiting step in the hydrolysis cycle, allowing Hsc70 to re-enter the ATP-bound state (26). The NEFs also govern the persistence and stability of the client complex (27). Most clients cannot fully fold when bound to Hsc70, such that dissociation is likely required for them to mature and function. Indeed, recent studies have shown that members of the Hsc70 family inhibit ligand binding to glucocorticoid receptor (28). In addition, this interaction keeps aggregation-prone clients, such as human telomerase repeat binding factor (hTRF1), on the folding trajectory by allowing proper secondary structures to form (29). Thus, Hsc70-client complexes seem to represent a “timing” mechanism for client folding, function and turnover, possibly helping to ensure proper quality control. These observations have also focused attention on the protein-protein interaction between Hsc70 and BAG family proteins as potential targets for drug discovery (3). Indeed, allosteric inhibitors of this interaction trigger degradation of a subset of Dengue viral proteins (30) and some oncogenes (31).

Each member of the BAG family of co-chaperones contains a conserved BAG domain that is important for binding to Hsc70. The BAG domain folds into a three-helix bundle that interacts with the NBD of human paralogs, including Hsc70 (HSPA8) and the closely related Hsp72 (HSPA1A). Co-crystal structures of Hsc70NBD bound to BAG domains have shown that the protein-protein interaction involves a large, polar surface that spans the “top” of lobes I and II (32–35). These co-crystal structures have also suggested a mechanism in which BAGs “open” the lobes of the NBD, favoring release of ADP and re-binding to ATP. This mechanism is reminiscent of that used by structurally unrelated NEFs, such as prokaryotic GpE (36), although the details of the interaction and the surfaces involved can be different. Regardless of the identity of the NEF, conversion to the ATP-bound state would be expected to indirectly favor the release of clients from Hsc70, because this conformer has relatively weaker affinity (37). Consistent with that idea, full-length Bag1, Bag2, and Bag3 accelerate the release of a fluorescent client peptide from human Hsp72 and Hsc70 in vitro (38). Also, NEFs are known to release cholera toxin from inactive, chaperone-bound complexes in cells (39). However, other observations suggest that NEFs might release clients through a mechanism that is more complex. For example, Hsc70NBD binds to full-length Bag1 with an affinity of ~12 nM in the ATP-bound state by isothermal calorimetry (ITC), but it only binds with an affinity of 95 nM to a truncated BAG domain under the same conditions (38). This result suggests that a secondary binding site might also contribute.

While studying the interaction between human Hsc70 and Bag1/3 in more detail, we made the surprising discovery that truncated variants, composed of only the BAG domain, are poor at promoting release of bound clients. This result suggested the possibility that, in the human system, release of ADP and client may not necessarily be linked. Using in vitro assays for nucleotide and client release, we characterized a non-canonical interaction between Bag1/3 and the Hsc70NBD that is essential for client release. This secondary contact was confirmed using an NMR-based binding assay and a truncated form of Bag3 that lacks the BAG domain. Together, these results support a revised model in which members of the BAG family of co-chaperones use two separate domains to promote release of nucleotide and clients from Hsc70.

Results

Isolated BAG Domains Promote Release of Nucleotide, but Not Model Client—In this study, we focused on the interaction of human Hsc70 (HSPA8) with Bag1 and Bag3. These systems were chosen because the Hsc70-Bag1 complex has been implicated in directing clients to the ubiquitin-proteasome system (UPS) (40), while the Hsc70-Bag3 pair links some clients to the macroautophagy pathway (41). Thus, these co-chaperones are some of the best-characterized BAG family members and they seem to be important in guiding client fate decisions under some conditions, such as antigen presentation (42). Further, the affinities of both protein-protein interactions are similar (Bag1 $K_d \sim 12 \text{nM}$; Bag3 $K_d \sim 10 \text{nM}$; binding to Hsc70NBD in the ATP-bound form by ITC) (38). To understand if nucleotide and client release activities are separable, we used two fluorescence polarization (FP) assays that have been previously developed (38). In the first, we saturated Hsc70 with a fluorescent nucleotide (6-(6-amino)hexyl-ATP-5-FAM; ATP-FAM) and then measured nucleotide release after addition of BAG protein. ATP and ADP have similar $K_d$ values in this platform (38), so it is often used to measure nucleotide release. In the second assay, a fluorescent client binds to the well-characterized HLA peptide (FAM-RENILRALRY; FAM-HLA) (43) was loaded into Hsc70 and its release by BAG proteins was measured. To make this assay especially sensitive to NEF activity, binding is carried out under conditions of high ADP (1 mM), such that the tight-binding conformer of Hsc70 is purposefully favored. In the first experiments, we compared the activities of full-length Bag1 and Bag3 to that of truncated versions composed of only the BAG domain (termed Bag1C and Bag3C, residues 107–219 and Bag3C (residues 421–499); Fig. 1A). In the nucleotide release assay, all of the proteins had approximately equivalent activity ($EC_{50}$ values ~0.4 to 0.7 $\mu$m; Fig. 1B), consistent with the reported importance of the BAG domain in ADP release (38). However, we found that Bag1C and Bag3C had only very weak ability to promote client release (EC_{50} > 10 $\mu$m; Fig. 1B). This was an unexpected result, which suggested that regions outside the BAG domain might be essential for this process.

Point Mutations in the BAG Domain Prevent Nucleotide Release, but Not Client Release—To disrupt binding of the BAG domains to Hsc70 with greater precision, we examined the co-crystal structure of Bag1’s BAG domain bound to Hsc70NBD (PDB = 1HX1) (34). Residues in Bag1 that are predicted to be involved in key contacts were then selected for mutation, including D208A, R193A, and K194A (Fig. 2A). There is no reported co-crystal structure of Hsc70NBD with the BAG domain of Bag3, so we used primary sequence alignment (supplemental Fig. S1), intuition and homology models to select E455K, R480A, and R481A for mutation. We found that the single point mutations had relatively modest effects on affinity by themselves (see below), so we also expressed and purified the double mutants: Bag1 (R193A,K194A) and Bag3 (R480A,R481A). The mutant proteins were all folded normally, as judged by circular dichroism (Fig. 2B). Using a flow cytom-
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(A) Partial domain architecture of Bag1 and Bag3

(B) BAG domain alone cannot promote client release

FIGURE 1. Isolated BAG domains promote release of nucleotide, but not client, from Hsc70. A, a partial domain architecture of human Bag1 and Bag3, highlighting the location of the BAG domain. Some known domains are not indicated for clarity. Bag1C and Bag3C are the isolated BAG domains. B, full-length Bag1/3, but not Bag1C or Bag3C, strongly promote release of a fluorescent client from Hsc70. The results are the average of four independent experiments performed in triplicate each. The error bars represent S.D. All of the NEFs were able to promote nucleotide release, as previously reported (see text).

single mutants, Bag1 D208A and Bag3 E455K, were still able to stimulate Hsc70, but only at high concentrations and the inhibitory effect was largely abolished (at least under the concentrations attainable in these platforms). The double mutants were not able to impact Hsc70 functions at all, consistent with the idea that they no longer have functional BAG domains.

Next, we asked whether the mutants could release nucleotide and/or client from the Hsc70-client complex using the FP assays. As expected, Bag1 (R193A,K194A) and Bag3 (R480A, R481A) were only able to weakly promote release of nucleotide (EC50 > 10 μM; Fig. 3A), likely because their BAG domains were compromised. However, these mutants were normal in their ability to release client (EC50 ~ 1 to 2 μM; Fig. 3B). The results with Bag1 (R193A,K194A) were particularly important because this double mutant had no measurable affinity for Hsc70NBD (KD > 500 μM; see Fig. 2C) and, thus, any remaining activity in client release assays must originate from a non-canonical interaction (meaning an interaction that does not involve the BAG domain). To further explore this model in Bag3, we found that deletion of the BAG domain created a co-chaperone (Bag3ΔBAG; Fig. 3C) that had normal activity in the client release assay (EC50 ~ 2 μM; Fig. 3D), but not nucleotide release assay (EC50 > 10 μM; Fig. 3E). Together, these results suggested that independent regions might be responsible for the ability of Bag1 and Bag3 to promote nucleotide and client release.

The Non-canonical Interaction of Bag3 with Hsc70 Occurs in the SBD—To understand which domain of Hsc70 might be required for this putative, non-canonical interaction, we created a truncated Hsc70 construct that lacked the NBD (Hsc70SBD; Fig. 4A). We found that the FAM-HLA tracer had a relatively poor signal (< 50 mP) upon binding to Hsc70SBD in the FP assay (data not shown), so we switched to a related tracer, FAM-LVEALY that is based on the well-known client peptide from microtubule-binding protein tau (10, 45). As expected, the FAM-LVEAVY tracer bound both full-length Hsc70 and Hsc70SBD with similar apparent affinity (Fig. 4B). Using this assay, we found that Bag1 and Bag3 accelerate release of the FAM-LVEAVY tracer from Hsc70SBD (Bag 1 EC50 ~ 3 μM and Bag3 EC50 ~ 1 μM; Fig. 4C). Moreover, we found that Bag1 (R193A,K194A), Bag3 (R480A, R481A), and Bag3ΔBAG all had similar activity as the wild type, full-length versions. Together, these results suggest that the non-canonical interaction does not require the NBD.

To further understand whether the non-canonical interaction occurs in the SBD, we wanted to develop a distinct biophysical assay using NMR. Toward that goal, we expressed and purified a 13C,15N-labeled truncation of Hsc70 that was composed of only the β sandwich subdomain (Hsc70 SBD β; residues 395–508; see “Materials and Methods” for a description of how this construct was chosen and see supplemental Figs. S2, S3, and S4 for additional spectra and controls). After denaturation and refolding, we found that this protein was monomeric and had the expected affinity for the model client peptide, NRLLLTG (KD ~ 10 μM; see supplemental Figs. S2, S3, and S4). We decided to carry out the binding studies at relatively low concentrations (11 μM) to favor biologically relevant interactions and to stay within the limits of Bag1/Bag3 solubility. However, under these conditions, the 15N-1H TROSY and/or HSQC...
spectra of Hsc70 SBD, especially in the apo-state, were not sufficient sensitive to be useful for ligand binding studies (data not shown). Hence, we opted for the more sensitive $^{13}$C-$^1$H HSQC spectra of the methyl groups, which yielded good spectra at low concentrations ($10 \, \mu$M; Fig. 4D).

As an initial control, we worked with a sample containing 80 $\mu$M $^{13}$C $^{15}$N Hsc70 SBD $\beta$ and confirmed that addition of a model client peptide, NRLLLTG (200 $\mu$M) caused extensive changes in the methyl spectra (supplemental Fig. S4). Specifically, extensive chemical shift changes were observed, including the appearance of new cross peaks in slow exchange. The appearance of new resonances, just as was seen in the $^{15}$N-$^1$H TROSY spectra (supplemental Fig. S2) suggest that the Hsc70 apo-SBD is dynamically disordered and that it rigidifies upon peptide binding. Similar, but less dramatic changes have been reported for the SBD of DnaK, the *Escherichia coli* Hsc70 homologue (6). The $^{13}$C-$^1$H HSQC spectra of Hsc70 SBD$\beta$ at 11 $\mu$M were, of course, less sensitive and we could not discern as many changes as seen in the spectra at 80 $\mu$M. However, NRLLLTG still caused significant chemical changes in a discrete number of methyl cross peaks (Fig. 4D) indicating binding with $\sim 10 \, \mu$M affinity. This peptide also induced a small number of new peaks to appear in slow exchange (for example, $\omega_1 \sim 18$ ppm and $\omega_2 \sim 1$ ppm).

Using this assay platform, we asked whether Bag3DEBAG or Bag3C could bind to Hsc70 SBD$\beta$. We found that addition of
Bag3ΔBAG (12 μM) induced new peaks (for example, $\omega_1 \approx 11$ to 13 and $\omega_2 \approx 0.25$) and strong CSPs, while nearly 3× more Bag3C (30 μM) was required to observe even modest and incomplete changes in the spectra (Fig. 4D). This result shows that Bag3ΔBAG, but not Bag3C, binds to Hsc70 SBD with an affinity of 10 μM or stronger. By analogy with the NRLLLTG peptide spectra, we assume that the chemical shift changes occur as a product of “rigidification” of Hsc70 SBD by binding to Bag3ΔBAG. Extensive characterization of the binding site or the changes will await future studies. For now, the NMR binding data serves to confirm that a region of Bag3 outside of the BAG domain binds to the Hsc70SBD.

**Discussion**

Bag1 and Bag3 Interact with Hsc70 through a Bi-dentate Mechanism and the Second Interaction Is Crucial for Client Release—Here, we report that a non-canonical interaction outside the BAG domain is essential for promoting client release in vitro. Most strikingly, isolated Bag1C or Bag3C were unable to promote client release, despite their normal activity in nucleotide release assays (see Fig. 1B). These results suggest a model in which Bag1 and Bag3 use at least two, distinct protein-protein interactions when binding to Hsc70 (Fig. 4E). Why are the activities of nucleotide release and client release separated in human Bag1 and Bag3? We speculate that, at least for some BAG family proteins, this bi-dentate interaction may provide an additional layer of regulation. In the canonical model, the interaction of the BAG domain with Hsc70NBD directly favors release of ADP and only indirectly triggers the release of clients after ATP re-binding and allosteric switching of the SBD. Indeed, even Bag1C is capable of promoting release of client from Hsp72 at high enough concentrations ($EC_{50} \approx 76 \mu M$) in vitro (38). However, we speculate that the rate or extent of this allosteric program may not be sufficient to drive function of the NEF-Hsc70 system under some conditions in vivo. Instead, the secondary, non-canonical interaction in the Hsc70SBD might allow for greater control over the kinetics and timing of client “hand-off”, perhaps coordinating the release to coincide with delivery to other pathways. This multi-pronged interaction might even position the other regions of Bag1 or Bag3 (e.g. the PXXP motif of Bag3) so that they can recruit effectors of macrorautophagy or the ubiquitin-proteasome system. Another possibility is that separating the two activities makes the system more responsive to cellular stress conditions. Under normal conditions, the levels of ATP are high compared with ADP, but this situation reverses in response to some types of stress or metabolic conditions. Thus, NEF function might sometimes be partly frustrated by re-binding of Hsc70 to ADP, disrupting progression through the nucleotide cycle. Under those conditions, the non-canonical contact might provide a salvage pathway to ensure dissociation of clients.

Is the Bi-dentate Interaction Conserved in Other NEFs?—It seems possible that other families of NEFs, such as GrpE and Hsp110, might also take advantage of bi-dentate binding mechanisms. For example, the crystal structure of GrpE bound to the NBD of DnaK shows a long helical coil extending from the site of the protein-protein interaction (46) that could possibly project toward the putative location of the SBD (which was truncated in the construct used for crystallography). While not directly parallel to the proposed mechanism of Bag1 and Bag3, the eukaryotic NEF, Hsp110, has spatially separated domains.
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FIGURE 4. The non-canonical interaction between Bag3 and Hsp72 occurs in the SBD. A, truncations of Hsc70 used in this study. B, client tracer interacts with both full-length Hsc70 and Hsc70SBD. C, Bag1, 3, double mutants and Bag3ΔBAG compete with client for binding to the Hsc70SBD. Results are the average of experiments performed in triplicate. Error is ± S.E. D, 600 MHz 13C-1H HSQC spectra of 11 H 6 15N Hsc70 (395–508) showing binding to a model client (NRLLTG), to Bag3ΔBAG and to Bag3C. In each overlay, the apo-protein spectrum is in blue, and the spectrum of the Hsc70 SBD domain in the presence of ligand is shown in red. The model client and Bag3ΔBAG cause chemical shift perturbations and appearances of new resonances in slow exchange at 1:1, whereas Bag3C only causes minor changes at 3:1. E, model for the bi-dentate mechanism of human Bag1 and Bag3. The identity and location of the SBD-binding site is not yet clear (see supplemental Fig. S1).
that are dedicated to binding Hsc70\textsubscript{NBD} and client (47, 48). It will be interesting to learn if other family members use bi-dentate mechanisms to coordinate nucleotide and client release. Such a model could create new opportunities for creating more selective inhibitors of NEF function. This possibility is important, given the diversity of functions played by NEFs (49). For example, it is not yet clear if this mechanism is conserved among the other members of the BAG family. Bag2 is important in linking some Hsc70 clients to the proteasome (50), while Bag5 has been implicated in alpha-synuclein homeostasis (51). Future work will be needed to see if these members of the co-chaperone family also bind Hsc70 through bi-dentate interactions.

Where on Bag1 and Bag3 Does the Non-canonical Interaction Occur?—Bag1 and Bag3 have little sequence identity (or similarity) beyond their conserved BAG domains (see supplemental Fig. S1). The NMR binding results suggested that the interaction could possibly involve a sequence that resembles a client because the changes in the \textsuperscript{13}C-\textsuperscript{1}H HSQC NMR spectra are somewhat similar after addition of either NRLLLTVG or Bag3\textsubscript{2ABAG} (see Fig. 4D). In that putative model, a “client-like” sequence in Bag1 or Bag3 might directly bind to the SBD’s cleft and release client by a competitive interaction. Along these lines, the fact that significant effects are observed by titration of \textsuperscript{13}C \textsuperscript{15}N Hsc70 SBD\beta (11 \textmu M) with approximately equivalent levels of Bag3\textsubscript{3ABAG} (12 \textmu M), suggests that the interaction is of relatively good affinity (estimated < 10 \textmu M). Such an affinity would mean that the non-canonical interaction could potentially compete with many Hsc70 clients, which often have \textit{K}_D values in the low micromolar range, especially if the BAG domain interaction with Hsc70\textsubscript{NBD} was able to increase local concentration. However, Bag1 and Bag3 only have a limited number of predicted, consensus Hsc70 binding sites (see supplemental Fig. S1). Each protein has one predicted site within the BAG domains, but these regions are expected to be partly occluded in the folded, three-helix bundle and; more importantly, Bag1C and Bag3C had poor activity in the client release assays (see Fig. 1). Outside the BAG domain, Bag3 only has one predicted consensus Hsc70 binding motif and Bag1 has none (see supplemental Fig. S1), suggesting that an alternative explanation may be required. One possibility is that the putative, second site does not have sufficient sequence similarity to known clients, so it is not detected by prediction algorithms (52). Alternatively, it seems possible that the non-canonical interaction might occur, at least in part, outside of the classic client-binding cleft rather than being directly competitive. In theory, this interaction could reposition the lid subdomain and/or the CTD to indirectly regulate client off-rates and accessibility of the binding cleft. At this point, the location of the binding site on Bag1 and Bag3 and where it binds on the SBD are unclear.

The Thermodynamics of the Bi-dentate Interaction Remains Uncertain—Another issue that remains to be resolved is the role of avidity in the interactions of full-length Hsc70 with Bag1 and Bag3 in cells. The apparent affinity of Bag3C for Hsc70 is \textasciitilde 30 \textmu M (in the apo state), as measured by either ITC or FCPIA, while the affinity for full-length Bag3 is 10-fold better (\textasciitilde 3 \textmu M) (38). That observation first suggested that there might be “missing” binding energy in the protein-protein complex. However, the apparent affinity of the Bag3\textsubscript{A}BAG protein for Hsc70 SBD\beta by NMR was \textasciitilde 10 \textmu M or less (see Fig. 4), which is significantly tighter than what would be minimally needed to account for the “missing” affinity. We were not able to measure the binding of Bag1C, Bag3C, or Bag3\textsubscript{A}BAG to Hsc70\textsubscript{NBD} by ITC or FCPIA (data not shown). Indeed, technical challenges, such as solubility, persistent client retention and signal/noise, have prevented collection of the affinity values for all of the comparisons (e.g. Hsc70 binding to Bag3\textsubscript{2A}BAG) that would be needed for a rigorous analysis of the thermodynamic contributions of the two sites to overall binding free energy. Regardless, the results reported here provide a strong impetus for the continued study of the non-canonical interactions between Hsc70 and BAG family members, as this contact appears to be necessary and sufficient for client release under some conditions.

Materials and Methods

**Protein Expression and Purification**—Human BAG1 (also called Bag1S), Bag3, Bag1C, Bag3C, Bag1, and Bag3 mutants, Bag3\textsubscript{2A}BAG, Hsc70 (HSPA8), Hsc70 SBD (residues 394–509) were expressed in BL21(DE3) cells as previously described (38). Following purification on Ni-NTA resin, overnight TEV cleavage to remove His tags, monQ (GE Healthcare) and size exclusion (SuperDex S200) columns, the stock proteins were dialyzed into HEPEs buffer (25 mM HEPEs, 5 mM MgCl\textsubscript{2}, 150 mM KCl pH 7.5). Hsc70 was also subject to 3 days of extensive dialysis to remove bound nucleotide and residual client.

**Nucleotide Release**—Fluorescence polarization (FP) experiments were carried out as previously described (38). Briefly, ATP-FAM (20 \textmu M; Jena Bioscience) dissociation from Hsc70 (1 \mu M) was measured by incubating BAGs for 10 min in black, round-bottom, low-volume 384-well plates (Corning) in assay buffer (100 mM Tris, 20 mM KCl, 6 mM MgCl\textsubscript{2} pH 7.4). After incubation, FP was measured (Ex 485 nm/Em 535 nm) using a SpectraMax M5 plate reader. Controls included wells lacking Hsc70. It has previously been reported that ATP and ADP have similar \textit{I}_C\textsubscript{50} values for the ATP-FAM tracer (38).

**Client Release**—Release of fluorescent FAM-HLA or FAM-LVEAVY from Hsc70 was measured by FP, as described (38, 53). Briefly, a mixture of Hsc70 (1 \mu M) and FAM-HLA (25 \textmu M; Anaspec) or FAM-LVEALY (25 \textmu M, Genscript) was incubated with BAG proteins for 30 min at room temperature in assay buffer (100 mM Tris, 20 mM KCl, 6 mM MgCl\textsubscript{2}, 1 \mu M ADP, pH 7.4). FP was measured (Ex 485 nm/Em 535 nm) using a SpectraMax M5 plate reader. Pilot studies showed that 30 min was sufficient to achieve equilibrium.

**Flow Cytometry Protein Interaction Assay (FCPIA)**—The assay procedure was adopted from previous reports (38). Briefly, Hsc70 biotinylated on lysine residues was immobilized (1h at room temperature) on streptavidin-coated polystyrene beads (Spherotech), in the presence of nucleotide (1 mM). After immobilization, beads were washed and then incubated with fluorescently labeled NEF protein. Binding was detected using a flow cytometer to measure median bead-associated fluorescence. Beads capped with biocytin were used as a negative control, and nonspecific binding to beads was subtracted.
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Chaperone Assays—The effects of BAG proteins on steady state ATPase activity were measured by malachite green assays, as previously described (38, 54). Effects on denatured firefly luciferase refolding were measured by luminescence assays, as previously described (38). Hsc70 was used at 1 μM and DnakA2 was added to 0.5 μM.

NMR Spectroscopy—In initial studies, we found that the NMR spectra of Hsc70 residues 395–646 to be unusable for binding studies because residues 600–646 are dynamically dis-ordered and obscure/dominant much of the NMR spectrum (55). We next tried the truncation 395–604, which contains only the β sandwich domain and alpha helical “lid”. However, we found that this construct, while monomeric, bound NROLLTG peptide with a K_D value above 100 μM (compared with the expected value of ~1 μM for full-length Hsc70) perhaps suggesting that part of the lid might partially occlude the SBD cleft in this truncation. Finally, the lid was removed, yielding the constructs 394–509 (Hsc70 SBD) and 395–508 (Hsc70 SBDβ), which were used interchangeably. We found 395–508 bound client peptides with a K_D < 10 μM, in addition to giving a well resolved NMR spectra (see supplemental Figs. S2–S4). This construct also yielded interpretable spectra (see below). Therefore, we elected to use 13C 15N Hsc70 SBDβ (residues 395–508 G3H6) for the NMR binding studies. No significant differences are expected between Hsc70 SBD and Hsc70 SBDβ, but both are included here for transparency.

13C 15N Hsc70 SBDβ (residues 395–508) G3H6 was expressed in E. coli in minimal medium supplemented with isotopically labeled amino acids. Previous studies indicated that the protein was co-purified with bound clients, which need to be removed using an extensive purification pro-"..."


via homology modelling and experimental data. *PLoS Comp. Biol.* 5, e1000475


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doi: 10.1074/jbc.M116.742502 originally published online July 29, 2016

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