

Binding of Human Nucleotide Exchange Factors to Heat Shock Protein 70 (Hsp70) Generates Functionally Distinct Complexes *in Vitro**[§]

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Background: There has been an expansion of the number of Hsp70 cochaperones in mammals, providing the opportunity for combinatorial assembly of permutations with specialized functions.

Results: We studied the chaperone activity of Hsp70 combined with four different NEFs and four J proteins.

Conclusion: Some combinations were active, whereas others were inactive.

Significance: Cochaperones appear to expand the functional diversity of Hsp70.

Proteins with Bcl2-associated anthanogene (BAG) domains act as nucleotide exchange factors (NEFs) for the molecular chaperone heat shock protein 70 (Hsp70). There are six BAG family NEFs in humans, and each is thought to link Hsp70 to a distinct cellular pathway. However, little is known about how the NEFs compete for binding to Hsp70 or how they might differentially shape its biochemical activities. Toward these questions, we measured the binding of human Hsp72 (HSPA1A) to BAG1, BAG2, BAG3, and the unrelated NEF Hsp105. These studies revealed a clear hierarchy of affinities: BAG3 > BAG1 > Hsp105 ≫ BAG2. All of the NEFs competed for binding to Hsp70, and their relative affinity values predicted their potency in nucleotide and peptide release assays. Finally, we combined the Hsp70-NEF pairs with cochaperones of the J protein family (DnaJA1, DnaJA2, DnaJB1, and DnaJB4) to generate 16 permutations. The activity of the combinations in ATPase and luciferase refolding assays were dependent on the identity and stoichiometry of both the J protein and NEF so that some combinations were potent chaperones, whereas others were inactive. Given the number and diversity of cochaperones in mammals, it is likely that combinatorial assembly could generate a large number of distinct permutations.

Heat shock protein 70 (Hsp70) belongs to a ubiquitous and abundant family of molecular chaperones that regulates protein quality control and homeostasis (1, 2). Members of this family are thought to play key roles in virtually every cellular process that involves proteins, including folding, stabilization, trafficking, and turnover. Accordingly, Hsp70 has become an attractive drug target for neurodegenerative and hyperproliferative disorders (3, 4). However, it is difficult to envision strat-

egies for selectively inhibiting its pathobiology without impacting its essential roles (5, 6). To help guide this process, there is an interest in better understanding how Hsp70 is recruited into its various functions.

Hsp70 is a 70-kDa protein that consists of two domains: an N-terminal nucleotide binding domain (NBD)² responsible for binding and hydrolyzing ATP and a C-terminal substrate-binding domain (SBD) that binds to “client” proteins. The two domains are allosterically coupled so that, when ATP is bound to the NBD, the SBD binds relatively weakly to clients (7). When ADP is bound in the NBD, a conformational change enhances the affinity of the SBD for clients by slowing the off-rate (8, 9). The clients of Hsp70 include a wide range of unfolded, misfolded, and partially folded proteins (10, 11). Indeed, Hsp70 has little ability to discriminate between polypeptide sequences (12), and it is possible that there may be a few proteins (or cellular processes) that evade an interaction with Hsp70 at some stage (13, 14).

A key insight into how Hsp70 might be able to “juggle” its multiple functions comes from studies on cochaperones (6). Cochaperones, including the J proteins and the nucleotide exchange factors (NEFs), interact with Hsp70 and guide its various activities. Specifically, the J proteins are a family of cochaperones that bind to Hsp70 in a region between the NBD and SBD (15). This interaction stimulates ATP hydrolysis and promotes client binding (16). In addition, some J proteins interact with clients directly. Thus, they are believed to recruit proteins to the Hsp70 system (17). Conversely, the NEFs are cochaperones that bind the NBD of Hsp70 to accelerate ADP and client release (18). Some of the NEFs act as scaffolding proteins, linking Hsp70 and its clients to a variety of cellular pathways (19). Thus, the cochaperones of Hsp70 are thought to “tune” the

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² The abbreviations used are: NBD, nucleotide binding domain; SBD, substrate binding domain; NEF, nucleotide exchange factor; BAG, Bcl2-associated anthanogene; Ni-NTA, nickel-nitrilotriacetic acid; ATP-FAM, N⁶-(6-Amino)hexyl-ATP-5-FAM; FCPIA, flow cytometry protein interaction assay; ITC, isothermal calorimetry.

enzymatic activity of the chaperone and help guide its interactions with protein clients and other cellular factors.

Much of our mechanistic knowledge of Hsp70 function comes from studies using the *Escherichia coli* orthologs, which include a single Hsp70 (DnaK), a J protein (DnaJ), and a NEF (GrpE). Although the major components of the eukaryotic system are conserved, the diversity of the system has been greatly expanded through evolution. For example, the human genome contains more than 10 Hsp70s, 13 NEFs, and at least 41 J proteins (17). When compared with the prokaryotic system, this increase in potential partners has generated an enormous number of possible combinations. Some of the reasons for this expansion are clear. For example, there are chaperone and cochaperone components designated for localization in the endoplasmic reticulum and mitochondrion (2). However, another pressure propelling this evolutionary expansion appears to be functional diversification. Deletion of individual, cytoplasmically expressed J protein genes in yeast often produces a phenotype (20–22), suggesting that they are not redundant (17, 23). In mammals, auxilin is a J protein that is exclusively dedicated to helping Hsp70 dissociate clathrin triskelions (24). Other J proteins are unable to compensate for loss of auxilin, suggesting that some cochaperones may have “evolved” to recruit Hsp70s into specific niche functions.

This concept of functional specialization is further exemplified by the human NEFs, especially the BAG domain proteins (25). Since the identification of BAG1 (26, 27), six members of the BAG family (*i.e.* BAG1–6) have been identified on the basis of an ~100-amino acid BAG domain. The BAG domain is thought to promote nucleotide release by binding to the NBD of Hsp70 (Hsp70_{NBD}). This hypothesis rests on the basis of structures of human Hsc70_{NBD} in complex with the BAG domains of BAG1 or BAG2, which suggest that the cochaperones may help “open” the nucleotide-binding cleft to assist ADP dissociation (28–30). In addition to their shared BAG domain, the members of the BAG family have additional domains with specialized functions (31). BAG1, for example, has an ubiquitin-like (UBL) domain that targets Hsp70 clients to the proteasome (32–34). BAG1 also binds to the antiapoptotic kinase Raf1, and it works with Hsp70 to stabilize that protein in cancer (35). Conversely, BAG2 has been associated with promoting the degradation of large aggregates, such as phosphorylated Tau (36). BAG3 has multiple protein-protein interaction motifs that link the Hsp70-BAG3 complex to the small heat shock proteins Hsp20 and Hsp22, the signaling molecule PLC- γ , 14-3-3 proteins, and the autophagy pathway (37–40). Thus, the “choice” of which BAG protein is bound to Hsp70 appears to help determine what will happen to the Hsp70-bound client. In this context, it becomes important to understand the factors that guide the interactions between Hsp70 and these cochaperones.

Here we have explored how the major cytoplasmic Hsp70 family members Hsp72 (HSPA1A) and Hsc70 (HSPA8) interact with the three BAG family members that have been most closely linked to chaperone functions: BAG1, BAG2, and BAG3. We also measured the binding of Hsp72 to Hsp105 α , which belongs to an evolutionary distinct group of NEFs (41). We found that these cochaperones have an apparent binding hierarchy of BAG3 > BAG1 > Hsp105 \gg BAG2. The NEF-

Hsp70 interactions were sensitive to nucleotide status, with the tightest interactions observed when Hsp72 was nucleotide-free (*e.g.* apo). All of the BAG proteins competed for binding to Hsp72, and they accelerated nucleotide and substrate release in the relative order expected from their affinities. To understand how this hierarchical binding might influence chaperone functions, we reconstituted Hsp72 with the four NEFs and the four major cytosolic J proteins DnaJA1, DnaJA2, DnaJB1, and DnaJB4. Using ATP hydrolysis and luciferase refolding assays, we found that some of the permutations were strongly active, whereas other combinations were inactive. These results show how the biochemical properties of mammalian Hsp70s might be diversified by combinatorial assembly with cochaperones.

MATERIALS AND METHODS

Recombinant Protein Production—Human BAG1S (referred to as BAG1 throughout), BAG2, and BAG3 were subcloned into pMCSG7 from cDNA using ligation-independent cloning (42), and the sequences were confirmed by DNA sequencing at the University of Michigan DNA Sequencing Core. The Hsp105 α construct was a gift from Xiaodong Wang (University of Toledo), and the BAG1C construct was a gift from Jason Young (McGill). Constructs were transformed into BL21(DE3) cells, and single colonies were used to inoculate Terrific Broth containing ampicillin (50 μ g/ml). Cultures were grown at 37 °C for 5 h, cooled to 20 °C, and induced overnight with 200 μ M isopropyl 1-thio- β -D-galactopyranoside. BAG1S-, BAG1C-, and BAG2-expressing cells were pelleted, resuspended in His binding buffer (50 mM Tris, 300 mM NaCl, and 10 mM imidazole (pH 8.0)) and protease inhibitor tablets (Roche), and then sonicated. Supernatants were incubated with Ni-NTA resin for 2 h at 4 °C, washed with binding buffer, His washing buffer (50 mM Tris, 300 mM NaCl, and 30 mM imidazole (pH 8.0)), and finally eluted with His elution buffer (50 mM Tris, 300 mM NaCl, and 300 mM imidazole (pH 8.0)). BAG3-expressing cells were pelleted, resuspended in BAG3 lysis buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, and 15 mM β -mercaptoethanol (pH 8.0)), sonicated, fractionated by ammonium sulfate precipitation (0–30% of saturation), resuspended in His binding buffer, and then applied to the Ni-NTA resin. After Ni-NTA columns, all proteins were subjected to tobacco etch virus (TEV) protease cleavage overnight and dialyzed into MonoQ buffer A (20 mM HEPES, 10 mM NaCl, and 15 mM β -ME (pH 7.6)). Proteins were applied to a MonoQ column (GE Healthcare) and eluted by a linear gradient of MonoQ buffer B (buffer A + 1 M NaCl). Fractions were concentrated and applied to a Superdex S200 (GE Healthcare) size exclusion column in BAG buffer (25 mM HEPES, 5 mM MgCl₂, and 150 mM KCl (pH 7.5)). DnaJA1, DnaJA2, DnaJB1, and DnaJB4 were purified using an Ni-NTA column, followed by overnight TEV cleavage of the His tag and gel filtration on a Superdex S200. Hsp72, Hsp72_{NBD}, and Hsc70 were purified as described elsewhere (43). Hsp105 α was purified using Ni-NTA resin (as described above), the His tag was removed by overnight incubation with TEV protease, and the protein was dialyzed into His binding buffer and then subjected to a second Ni-NTA column. The flow-through was concentrated using Millipore Amicon filters, and protein was exchanged into BAG buffer.

Binding of Human Nucleotide Exchange Factors to Hsp70

To make apo-Hsp72, the protein underwent extensive dialysis: day 1 (25 mM HEPES, 100 mM NaCl, and 5 mM EDTA (pH 7.5)), day 2 (25 mM HEPES, 100 mM NaCl, and 1 mM EDTA (pH 7.5)), and day 3 (25 mM HEPES, 5 mM MgCl₂, and 10 mM KCl (pH 7.5)). NEFs were labeled with Alexa Fluor 488 5-SDP ester or Alexa Fluor 647 NHS ester (Invitrogen) according to the instructions of the supplier. Hsp72 was biotinylated using EZ-link NHS-Biotin (Thermo Scientific) according to the instructions of the supplier. After labeling, the proteins were subjected to gel filtration to remove any unreacted label. Average label incorporation was between 0.5 and 2.0 moles of label/mole of protein, as determined by measuring fluorescence and protein concentration ($A_{\text{max}} \times \text{molecular weight of protein} / [\text{protein}] \times \epsilon_{\text{dye}}$).

Flow Cytometry Protein Interaction Assay—The assay procedure was adopted from previous reports (44). Briefly, biotinylated Hsp72 was immobilized (1 h at room temperature) on streptavidin-coated polystyrene beads (Spherotech), with nucleotide (1 mM) present where indicated. After immobilization, beads were washed to remove any unbound protein and then incubated with labeled NEF protein at the indicated concentrations, with nucleotide where noted. Binding was detected using an Accuri™ C6 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 from the signal.

Isothermal Titration Calorimetry—NEFs and Hsp72_{NBD} were dialyzed overnight against ITC buffer (25 mM HEPES, 5 mM MgCl₂, and 10 mM KCl (pH 7.5)). Concentrations were determined using a BCA assay (Thermo Scientific), and the experiment was performed with a MicroCal VP-ITC (GE Healthcare) at 25 °C. Hsp72_{NBD} (100 μM) in the syringe was titrated into a 5–10 μM cell solution of NEF protein. Calorimetric parameters were calculated using Origin® 7.0 software and fit with a one-site binding model.

Fluorescence Polarization Assays—A fluorescent ATP analog, N⁶-(6-Amino)hexyl-ATP-5-FAM (ATP-FAM) (Jena Bioscience) was used to measure NEF-induced nucleotide dissociation from Hsp72. In black, round-bottom, low-volume, 384-well plates (Corning), 1 μM Hsp72 and 20 nM ATP-FAM were incubated with varying concentrations of BAG protein for 10 min at room temperature in assay buffer (100 mM Tris, 20 mM KCl, and 6 mM MgCl₂ (pH 7.4)). After incubation, fluorescence polarization was measured (excitation, 485 nm; emission, 535 nm) using a SpectraMax M5 plate reader. For substrate binding/dissociation, a commercially available fluorescent peptide, FAM-HLA (AnaSpec), was used as described (45). Briefly, 1 μM Hsp72 and 25 nM FAM-HLA were incubated with varying concentrations of BAG protein for 30 min at room temperature in assay buffer (100 mM Tris, 20 mM KCl, and 6 mM MgCl₂ (pH 7.4)). After incubation, fluorescence polarization was measured (excitation, 485 nm; emission, 535 nm) using a SpectraMax M5 plate reader.

Malachite Green ATPase Assay—Experiments were performed according to previous protocols (46, 47). Briefly, Hsp72 (1 μM) and various concentrations of NEF and/or J protein were added to clear 96-well plates, and the reactions were initiated with the addition of ATP (1 mM). The reactions proceeded for 1 h at 37 °C, were developed with malachite green reagent and

quenched with sodium citrate, and then plate absorbance was measured at 620 nm. A phosphate standard curve was used to calculate pmol ATP/μM Hsp72/min.

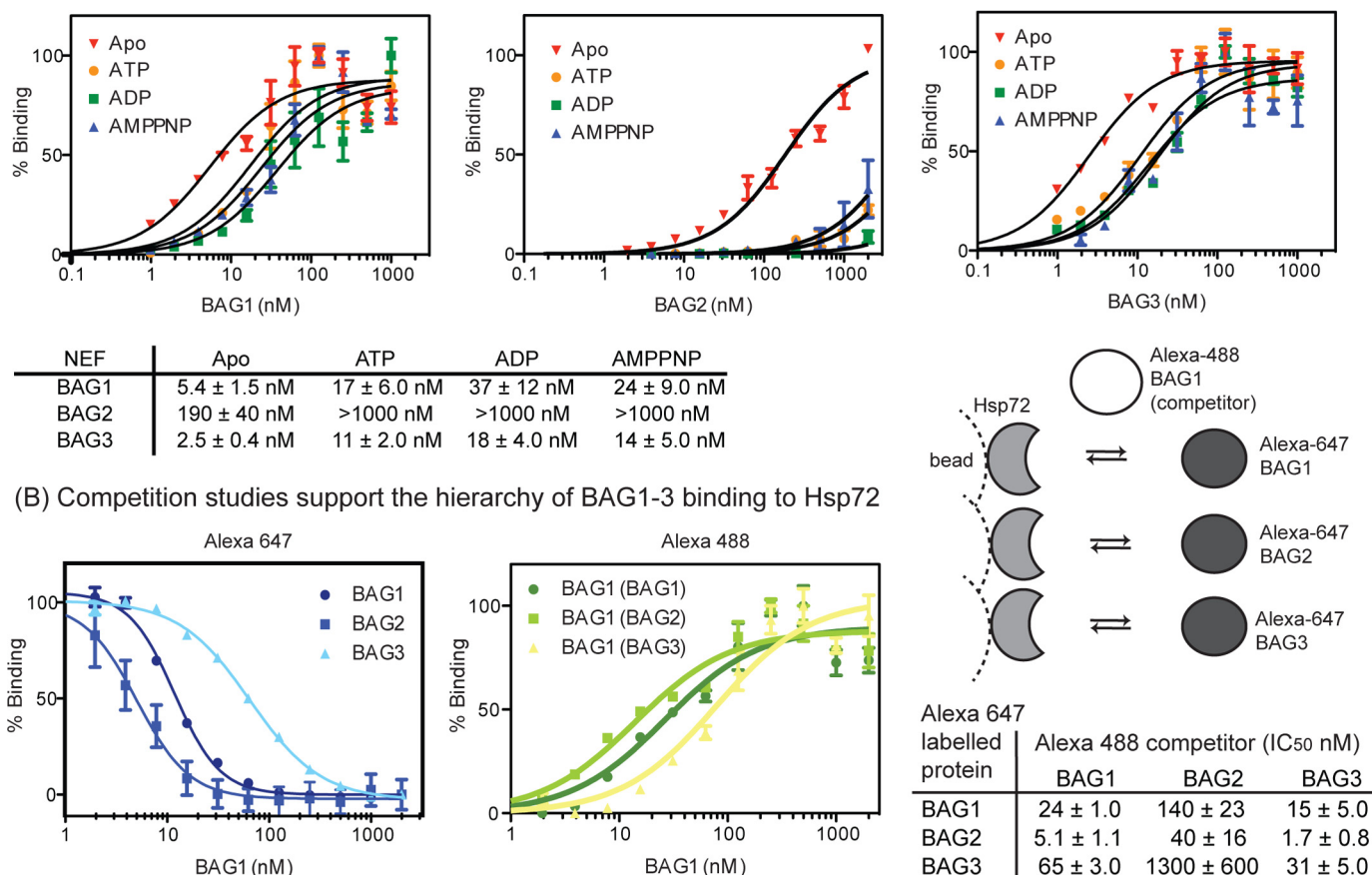
Luciferase Refolding Assay—Experiments were performed as described previously (48). In brief, luciferase (Promega) was denatured in 6 M GnHCl for 1 h at room temperature and then diluted into a working solution of Hsp72 in buffer containing an ATP regenerating system (23 mM HEPES, 120 mM KAc, 1.2 mM MgAc, 15 mM DTT, 61 mM creatine phosphate, 35 units/ml creatine kinase, and 5 ng/μl BSA (pH 7.4)). Various concentrations of NEF and J protein were added, and the reaction was initiated with the addition of ATP (1 mM). Sodium phosphate (10 mM) was added as indicated. The assay proceeded for 1 h at 37 °C in white, 96-well plates, and luminescence was measured using SteadyGlo luminescence reagent (Promega).

RESULTS

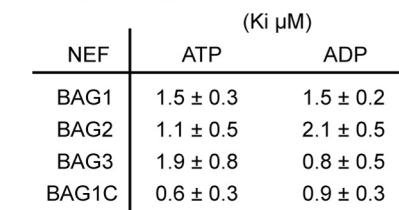
BAG Proteins Prefer Nucleotide-free Hsp70 and Exhibit a Hierarchy of Binding Affinities—To understand how BAG proteins regulate Hsp70 function, we first set out to determine how tightly they bind using two different platforms: a flow cytometry protein interaction assay (FCPIA) and isothermal titration calorimetry (ITC). In these studies, we were interested in whether BAG proteins might have similar or different affinities for Hsp70 and whether this affinity was dependent on the nucleotide status of Hsp70. Previous studies have shown that BAG1 has a better affinity for ATP-bound Hsp70 than ADP-bound Hsp70 (29, 49), but this property has not been systematically explored across all of the BAG proteins. For our FCPIA experiments, purified Hsp72 (HSPA1A) was biotinylated and immobilized on streptavidin-coated polystyrene beads. Solutions of fluorescently labeled BAG proteins were then incubated with the beads, and binding was detected using a flow cytometer. We found that BAG3 (11 ± 2 nM) had the tightest affinity for Hsp72 in the ATP-bound form, followed by BAG1 (17 ± 6 nM) and then BAG2 (>1000 nM) (Fig. 1A). The BAG proteins had a notably weaker affinity for ADP-Hsp70, with BAG3 binding with a K_D of 18 ± 4 nM and BAG1 at 37 ± 12 nM. Similar results were observed when ADP was replaced with the non-hydrolyzable nucleotide analog adenosine 5'-(β,γ-imino)-triphosphate (Fig. 1A). Surprisingly, we found that all three BAG proteins had their best affinity for apo-Hsp70, with the K_D values enhanced ~4-fold compared with the ATP-bound form. Together, these results demonstrate that all of the BAG proteins prefer the apo form of Hsp70 and that BAG3 binds tighter than BAG1 or BAG2.

Structural studies suggest that only one BAG protein can bind to Hsp70 at a time because they share a similar interaction surface on the NBD (29, 30). To test this model, we labeled each of the BAG proteins with either Alexa Fluor 647 or Alexa Fluor 488 and then used the Alexa 488-labeled samples to compete with the Alexa Fluor 647-labeled samples. In the FCPIA platform, we were able to measure both the loss of the Alexa Fluor 647 signal and the increase in bound Alexa Fluor 488-labeled protein (see schematic in Fig. 1B). The advantage of this approach is that we could simultaneously measure the release of the bound BAG protein and the binding of the competitor. Using this method, each BAG protein competed with itself and

(A) The affinity of Hsp72 for BAG1-3 is dependent on the identity of the BAG protein and nucleotide state



(B) Competition studies support the hierarchy of BAG1-3 binding to Hsp72



(C) Nucleotides weaken BAG1-3 binding to Hsp72

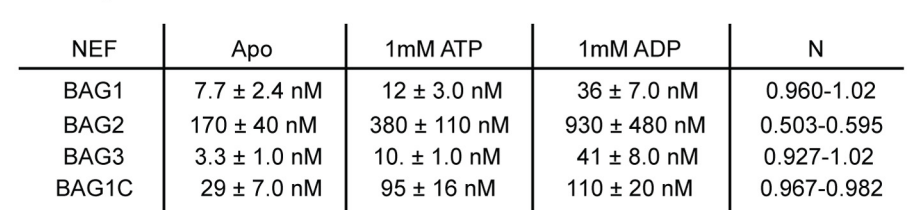


FIGURE 1. The affinity of Hsp70 for BAG1-3 is dependent on the identity of the BAG protein and the nucleotide state of Hsp70. *A*, measurement of the affinity of Hsp72 (HSPA1A) to BAG1-3 by FCPIA. Hsp72 was immobilized on beads, and binding to fluorescent BAG1-3 was measured. Experiments were performed in triplicate on three independent days with at least two different protein preparations. A representative result is shown. *Error bars* represent S.E. AMPPNP, adenosine 5'-(β , γ -imino)triphosphate. *B*, using the FCPIA platform, the relative ability of Alexa Fluor 488-labeled BAG1-3 to compete for binding with Alexa Fluor 674-labeled BAG1-3 was determined. A schematic of the method is shown. Experiments were performed in triplicate. *Error bars* represent S.E. *C*, binding of BAG1-3 and the truncated BAG domain of BAG1 (BAG1C) to purified Hsp72_{NBD} (residues 1-394) was measured by ITC. The results confirmed the relative hierarchy of affinity values. Note that BAG2 is a dimer. Thus, the N value of 0.5 suggests a complex of one BAG2 dimer per Hsp72_{NBD}. *D*, nucleotide displaces NEFs from Hsp72. The binding of BAG1-3 and BAG1C (100 nM) to Hsp72 was measured by FCPIA, and the inhibitory values (K_i) for ATP and ADP are shown.

with the other BAG proteins (Fig. 1*B*). Consistent with the previous results, BAG3 was the best competitor, followed by BAG1 and then BAG2. As a control, we attempted to displace BAG1 with the tetratricopeptide repeat protein CHIP. CHIP is known to bind Hsp70 in a distinct location at the C terminus (50, 51), so it would not be expected to interfere with binding of Hsp70 to BAG proteins. Consistent with this idea, CHIP could not compete with labeled BAG1 (data not shown). Because nucleotides appeared to weaken the interaction between Hsp72 and BAG1-3, we also specifically tested whether ATP or ADP might interrupt the protein-protein interactions using the

FCPIA approach. We found that both nucleotides released Hsp72 from BAG1-3, with IC₅₀ values of between 0.8 and 2.1 μ M (Fig. 1*C*).

Using ITC, we then confirmed the affinities of the BAG proteins for Hsp72 (Fig. 1*D*). These binding studies were performed using the NBD of Hsp72 (residues 1-394) because this region is thought to be sufficient for binding BAG proteins (30, 52), and it is more soluble in the ITC platform. We found that BAG3 bound apo-Hsp72_{NBD} with the tightest affinity (K_D = 3.3 ± 1.0 nM), followed by BAG1 (7.7 ± 2.4 nM) and BAG2 (170 ± 40 nM). These affinity values mirrored those obtained

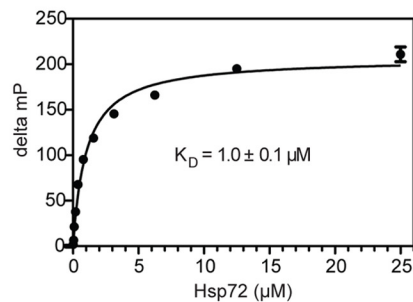
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using full-length Hsp70 in the FCPIA platform, suggesting that the NBD is indeed the only region of Hsp70 required for the interaction. To explore the minimal region of BAG1 required, we measured binding of Hsp72_{NBD} to the truncated BAG domain (BAG1C, residues 107–219). The affinities of BAG1C for Hsp72_{NBD} in the apo-, ATP- and ADP-bound states were uniformly weaker than the affinities of Hsp72 for full-length BAG1. For example, BAG1C bound ATP-Hsp72_{NBD} with an affinity of 95 ± 16 nM, whereas full-length BAG1 bound 8-fold tighter (12 ± 3 nM) (Fig. 1D). These results suggest that regions outside of the BAG domain contribute to binding Hsp72. Finally, the ITC studies also provided an estimate of the stoichiometry of the complexes. BAG1, BAG1C, and BAG3 all yielded N values of ~ 1 , suggesting the formation of a 1:1 complex with Hsp72_{NBD}, whereas BAG2 behaved as a dimer ($N \sim 0.5$), consistent with previous reports (30).

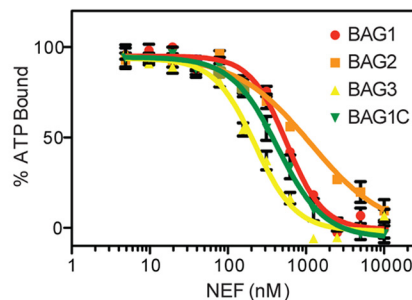
Collectively, these studies revealed that BAG proteins have a hierarchy of binding to Hsp72 and that nucleotide status is important in controlling their affinity. To test whether other Hsp70 family proteins share this characteristic, we repeated the FCPIA-based binding studies with the constitutive Hsp70, termed Hsc70 (HSPA8). The results were similar to those obtained with Hsp72, with BAG3 being the tightest-binding NEF and the apo state being the most amenable for binding BAG proteins (data not shown). Thus, these features appear to be conserved between the major cytoplasmic Hsp70 family members.

BAG Proteins Cause Nucleotide and Peptide Substrate Dissociation from Hsp70—Human BAG1 has been shown to promote the release of nucleotide and bound client proteins from Hsp70 (53–55), but the generality of this model has not been tested. Further, these activities have not been compared side by side to determine which BAG proteins might be the most potent NEFs. Toward those goals, we employed two fluorescence polarization assays that measure the release of fluorescent nucleotide (ATP-FAM) (56) and peptide substrate (HLA-FAM) (45, 57), respectively. First, we confirmed that ATP-FAM binds Hsp72 with an apparent K_D of 1.0 ± 0.1 μ M (Fig. 2A). Using this data, we selected a concentration of Hsp72 (1 μ M) and titrated with BAG proteins to determine an EC_{50} for nucleotide release. The results showed that the potency of BAG-induced nucleotide release correlated with their relative affinity values (Fig. 2B). Specifically, BAG3 was the most efficient NEF ($EC_{50} = 210 \pm 60$ nM), followed by BAG1 and BAG2 (630 ± 190 and 1040 ± 220 nM, respectively). BAG1C also acted as a NEF ($EC_{50} = 470 \pm 80$ nM), consistent with the importance of the BAG domain. Interestingly, BAG1C was not substantially worse than BAG1 in this context, suggesting that any contacts outside of the BAG domain are not relevant for nucleotide release. As controls, we attempted to use unrelated proteins as NEFs and found that none of them (J protein (DnaJA2), a model peptide client (NR peptide), or BSA) could promote nucleotide release ($EC_{50} > 10,000$ nM). However, ATP and ADP could compete with ATP-FAM, as expected (56). These results show that BAG proteins indeed function as NEFs for Hsp70 *in vitro* and, in general, that their relative potencies seem to be linked to their affinities for Hsp70.

(A) Binding of fluorescent nucleotide to Hsp72



(B) BAG1-3 act as nucleotide exchange factors



NEF	EC_{50} (nM)
BAG1	630 ± 190
BAG2	1040 ± 220
BAG3	210 ± 60
BAG1C	470 ± 80
ATP	2600 ± 1400
ADP	1900 ± 300
DnaJA2	$>10,000$
BSA	$>10,000$
NR Peptide	$>10,000$

FIGURE 2. **BAG1–3 promote nucleotide release from Hsp72.** A, ATP-FAM binding to Hsp72 as measured by fluorescence polarization. Experiments were performed in triplicate. Error bars represent S.E. B, BAG1–3 and the BAG1C domain promote release of ATP-FAM.

To investigate whether the BAG proteins also promote release of peptide substrates from Hsp70, we employed a fluorescently labeled model peptide (HLA-FAM) (45, 57). Hsp72 bound the probe with a K_D of 3.3 ± 1.6 μ M in the absence of added nucleotide, and the affinity increased to 0.27 ± 0.05 μ M in the presence of excess ADP (1 mM) (Fig. 3A). As expected, Hsp72_{NBD} was not able to bind HLA-FAM because it lacks the SBD (Fig. 3A). Using this platform, we titrated BAG1, BAG2, BAG3, and BAG1C into full-length Hsp72 (1 μ M + 1 mM ADP) and found that all of them could facilitate peptide release. In general, the relative potency values tracked with their apparent affinity values (Fig. 3B). However, BAG1C was ~ 40 -fold less efficient than its full-length counterpart, suggesting that regions outside of the BAG domain are important for release of HLA-FAM peptide from Hsp72. The control proteins BSA and CHIP were unable to accelerate substrate release, whereas NR peptide directly competed with the probe, as expected. Together, these results show that the BAG proteins promote release of substrates from Hsp72 and suggest that regions outside of the BAG domain might be important for this NEF activity.

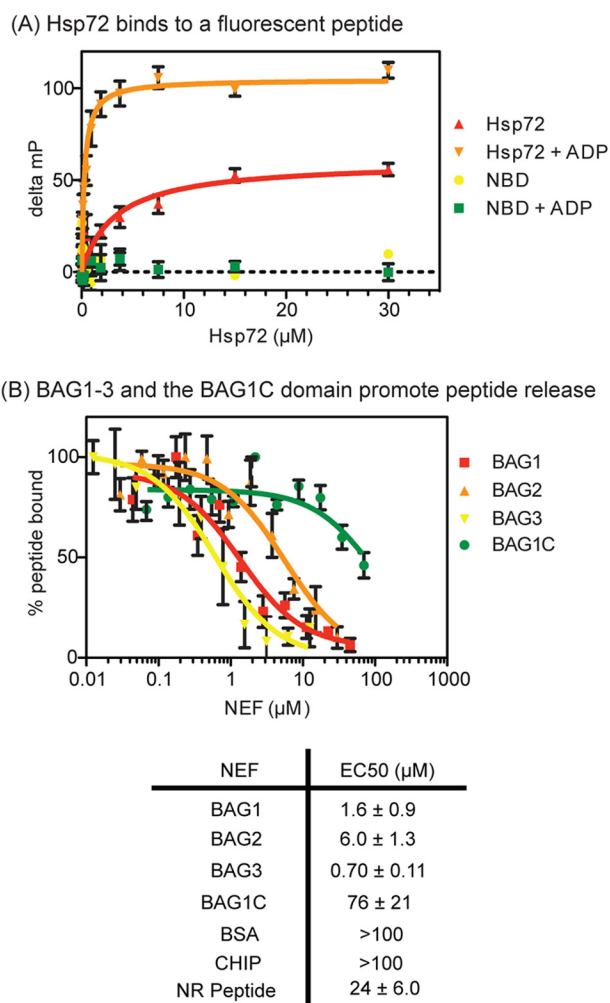


FIGURE 3. **BAG1–3 promote release of peptide clients from Hsp72.** *A*, binding of an HLA-FAM peptide to Hsp72 is dependent on nucleotide and the presence of the SBD, as measured by fluorescence polarization. Experiments were performed in triplicate. Error bars represent S.E. *B*, BAG1–3 and the BAG1C domain stimulate peptide release, with relative potency values (EC₅₀) that mirror their relative affinities.

Specific Ratios of BAG Proteins and J Proteins Combine to Influence Hsp70 ATPase Rates—Pioneering studies by Young and co-workers (21, 58) showed that Hsp70 is only able to fold denatured luciferase when combined with the J protein DnaJA2 but not DnaJA1. These results suggest that some combinations of Hsp70 with its cochaperones might have discrete biochemical functions *in vitro*, so we wondered how broadly this concept might be applied. The human J proteins are divided into three classes (A, B, and C) (17). The four major J proteins of the cytosol include two members of class A, DnaJA1 and DnaJA2, and two members of class B, DnaJB1 and DnaJB4 (59). Thus, to expand on the observations of Young and co-workers (21, 58), we combined Hsp72; BAG1–3; and DnaJA1, DnaJA2, DnaJB1, or DnaJB4 to generate 12 permutations. These combinations were then tested for their relative activity in functional assays that measure ATP turnover and luciferase refolding.

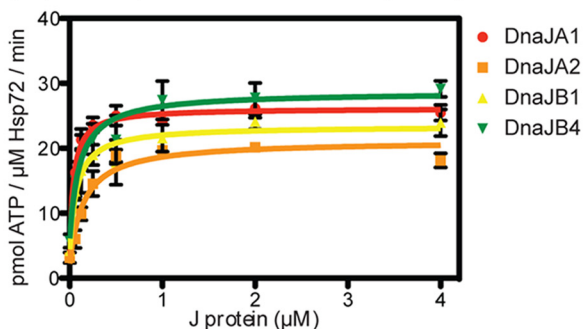
Although there have been extensive studies on the ability of prokaryotic J proteins to promote ATP turnover (16, 60), less is known about the human J proteins. DnaJA1 and DnaJA2 are known to accelerate nucleotide hydrolysis (16, 58), but this

property has not been explored for members of the B class, and their relative potencies are not yet clear. We found that DnaJA1, DnaJA2, DnaJB1, and DnaJB4 all stimulated the steady-state ATPase activity of Hsp72, as measured by malachite green assays (Fig. 4A). The potencies of all four J proteins were similar, supporting the presumption that they interact with Hsp72 through their highly conserved J domain in a similar manner (22, 61). None of the BAG proteins strongly stimulated the ATPase rate of Hsp72 in the absence of J protein (Fig. 4B), consistent with previous reports for a subset of these proteins (53, 62, 63). Using this benchmark, we then titrated Hsp72 (1 µM) with the four J proteins and the three BAG proteins and measured ATP turnover (supplemental Fig. 1). We found that low, substoichiometric concentrations of each BAG (e.g. 0.25 µM BAG1 or BAG3 or 0.125 µM BAG2) could promote the ATPase activity of each of the Hsp72-J protein pairs (Fig. 4B and supplemental Fig. 1). Increasing the levels of the BAG proteins (e.g. 4 µM BAG1 or BAG3 or 16 µM BAG2) tended to switch this behavior (Fig. 4C and supplemental Fig. 1). Specifically, high levels of the BAG proteins tended to inhibit ATPase activity, perhaps because they stabilize the apo form of Hsp72. However, in the case of BAG2, the extent of ATPase inhibition was dependent on the identity of the J protein. For example, BAG2 (16 µM) inhibited the ATPase activity of the Hsp72-DnaJA1 system, but it was synergistic with the Hsp72-DnaJA2 pair and neutral for the Hsp72-DnaJB4 pair. Together (Fig. 4D), these results provide evidence for specific combinations of Hsp72 and its cochaperones acting as biochemically distinct complexes.

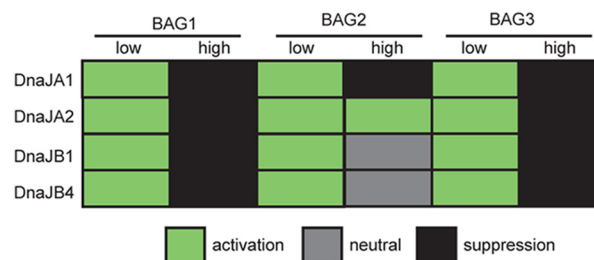
Combinations of Hsp72, J Proteins, and BAGs Generate Complexes with Distinct Chaperone Functions—The ability of Hsp70 to refold denatured clients, such as firefly luciferase, is a convenient *in vitro* method for estimating chaperone function. Hsp70 typically requires ATP and a J protein for this activity (64), and BAG1 has been shown to inhibit refolding in some studies (65). However, a systematic approach (in which the identity and stoichiometry of the cochaperones is varied) has not been reported. Toward that goal, we titrated Hsp72 with J proteins and BAGs and tested the ability of each permutation to rescue denatured firefly luciferase, as measured by recovered luminescence. Consistent with previous reports (21, 58), DnaJA1 was unable to promote luciferase folding by Hsp72 (1 µM) at any concentration tested (Fig. 5A). Addition of BAG proteins was unable to rescue this defect, suggesting that DnaJA1 is not competent for client folding even in the presence of NEFs. DnaJA2 and DnaJB4 both promoted refolding with maximal activity between 0.5 and 1.0 µM (Fig. 5A), whereas DnaJB1 was slightly less potent (maximal activity at 1.0 to 2.0 µM). Thus, although all of the J proteins are able to stimulate nucleotide hydrolysis to an identical extent, they vary in their ability to promote folding. This result is consistent with the idea that ATPase rate and the extent of client refolding are not directly linked (66). At higher concentrations of J proteins, refolding was inhibited, likely because the J proteins bind to luciferase and interfere with the folding process (13). Next, we tested the effects of inorganic phosphate on the activity of the J proteins. Previous work by Gassler *et al.* (68) has shown that physiological concentrations of phosphate suppress the refold-

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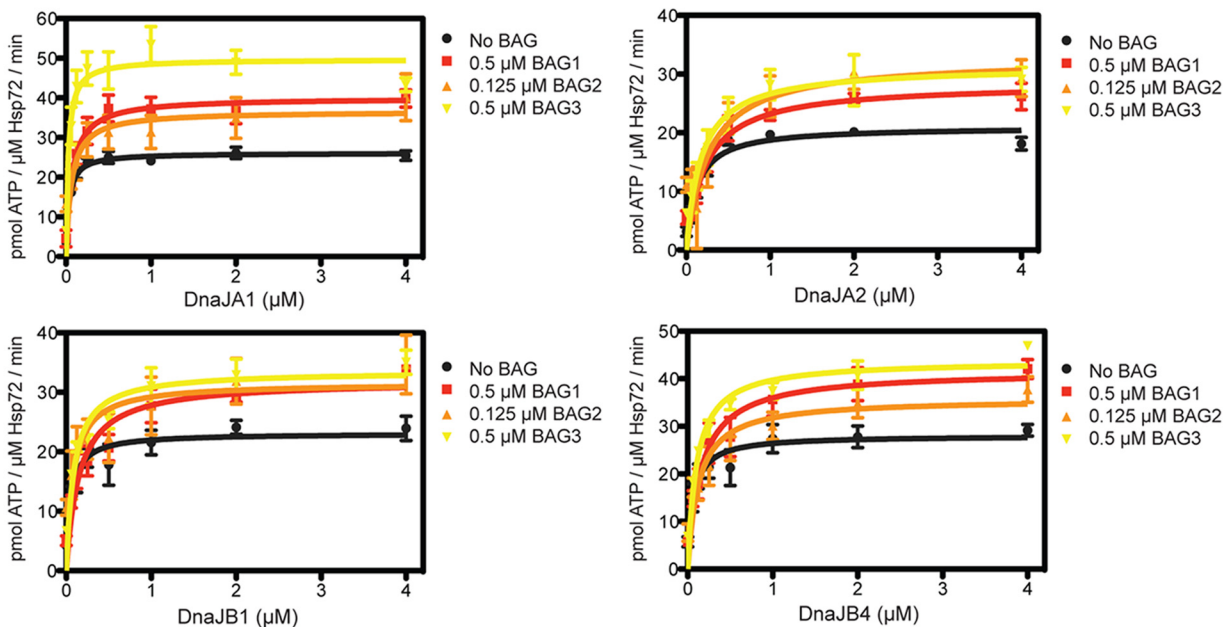
(A) J proteins promote ATPase activity in Hsp72



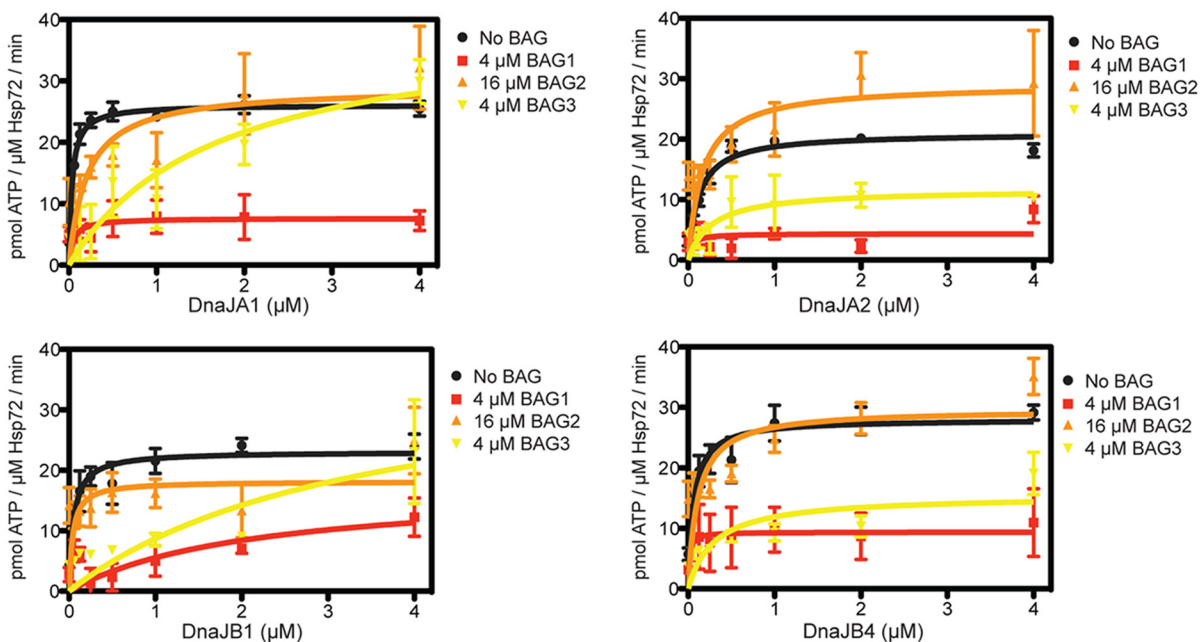
(D) Summary of the effects of BAG1-3 on ATPase activity



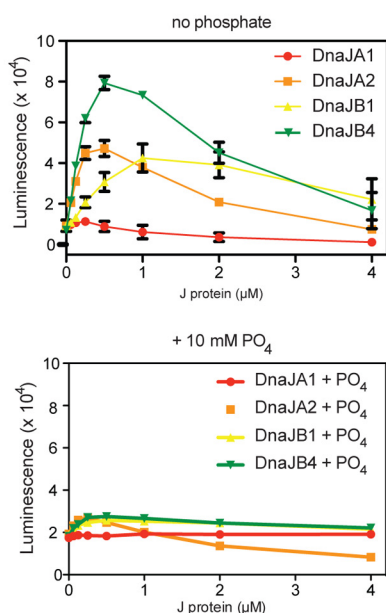
(B) BAG1-3 collaborate with J proteins to stimulate ATPase activity at low concentrations



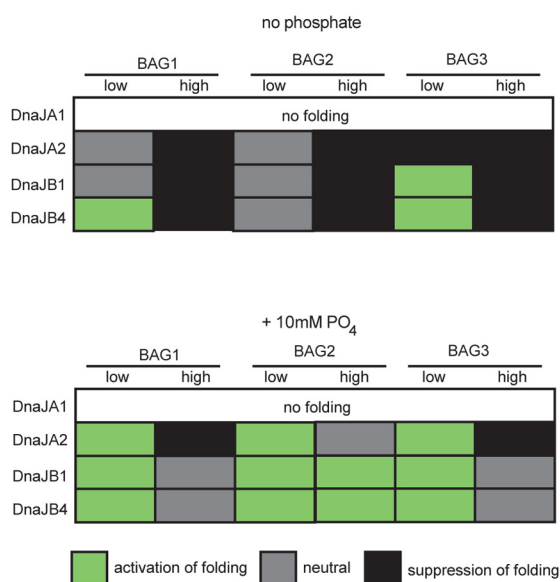
(C) BAG1-3 partially inhibit ATPase activity at high concentrations



(A) J proteins have differing abilities to work with Hsp72 in refolding denatured luciferase



(C) Summary of folding-competent Hsp72 combinations



(B) BAG1-3 either promote or inhibit luciferase refolding, depending on their concentration, the identity of the J protein and the presence of Pi.

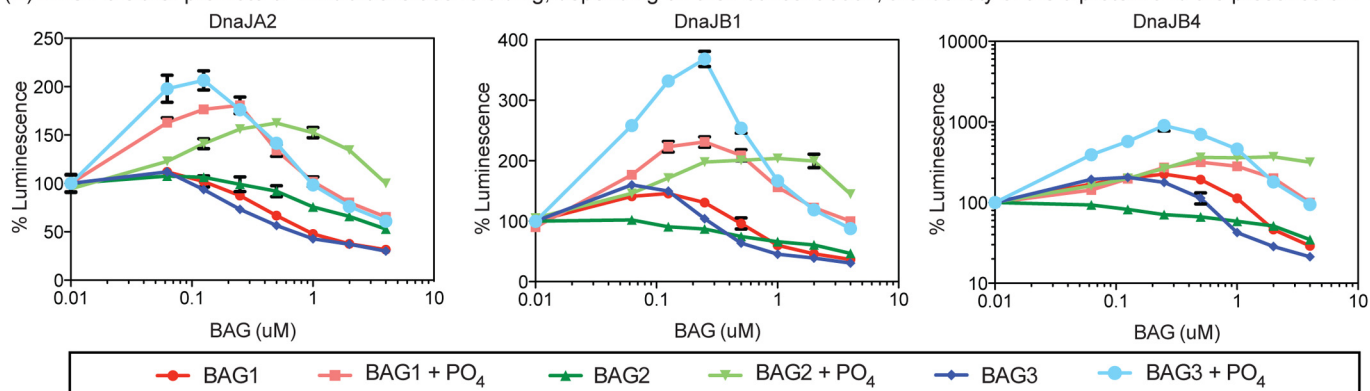


FIGURE 5. BAG1-3 coordinate with J proteins to tune luciferase refolding. *A*, individual J proteins have different profiles of luciferase refolding that is suppressed in the presence of phosphate (10 mM). Firefly luciferase was chemically denatured; mixed with Hsp72, J protein, and ATP; and then recovered luminescence was measured. The presence of phosphate (10 mM) did not influence the luminescence signal, as shown by the standard curve. Experiments were performed in triplicate. *Error bars* represent S.E. *B*, mixtures of Hsp72, J proteins, and BAG1-3 were used to refold luciferase. See [supplemental Fig. 2](#) for the full results. For clarity, only the effects of varying BAG1-3 in the presence and absence of 10 mM phosphate are shown. J protein concentrations were 0.5 μM DnaJA2, 2 μM DnaJB1, and 1 μM DnaJB4. All experiments were performed in independent triplicates. *Error bars* represent S.E. The y axis is normalized so that 100% luminescence is the amount of signal observed in the absence of NEF. *C*, schematic overview of the effects of BAG1-3 and J proteins on refolding activity.

ing activity of DnaJB1 and Hsc70. Indeed, we found that 10 mM sodium phosphate strongly suppressed the ability of DnaJA1, DnaJB1, and DnaJB4 to promote luciferase refolding (Fig. 5A). Phosphate (10 mM) itself had no effect on the assay (data not shown), simplifying the interpretation of these results.

To explore the specific effects of BAG1-3 on the refolding activity of these complexes, we combined the chaperones and cochaperones in the presence and absence of phosphate. In the absence of phosphate, we found that low levels of BAG1, BAG2, or BAG3 suppressed refolding by the Hsp72-DnaJA2 pair,

whereas these same levels of BAG1 and BAG3 could stimulate the refolding activity of the Hsp72-DnaJB1 pair (Figs. 5B and [supplemental Fig. 2](#)). Thus, certain BAG proteins worked synergistically with some J proteins but not others. In addition, the stoichiometry of the BAG proteins appeared to be important. This conclusion had been suggested by previous work on BAG1 (68), and our results suggest that it is a general property. For example, low concentrations (0.05–0.1 μM) of BAG3 enhanced the activity of the Hsp72-DnaJB1 pair, whereas higher levels of BAG3 (> 0.5 μM) were strongly inhibitory (Fig. 5B and

FIGURE 4. BAG1-3 stimulate ATPase activity in collaboration with J proteins. *A*, all four J proteins stimulate the ATPase activity of Hsp72, as measured by malachite green assays. The ATPase activity of Hsp72 was tested at various concentrations of J protein and NEF. BAG proteins stimulated ATP turnover at low levels (*B*) but inhibited this activity at increased concentrations (*C*). All experiments were performed in independent triplicates. *Error bars* represent S.E. Only representative BAG concentrations are shown, and the full dataset can be found in [supplemental Fig. 1](#). *D*, schematic overview of the effects of BAG1-3 and J proteins on ATPase activity.

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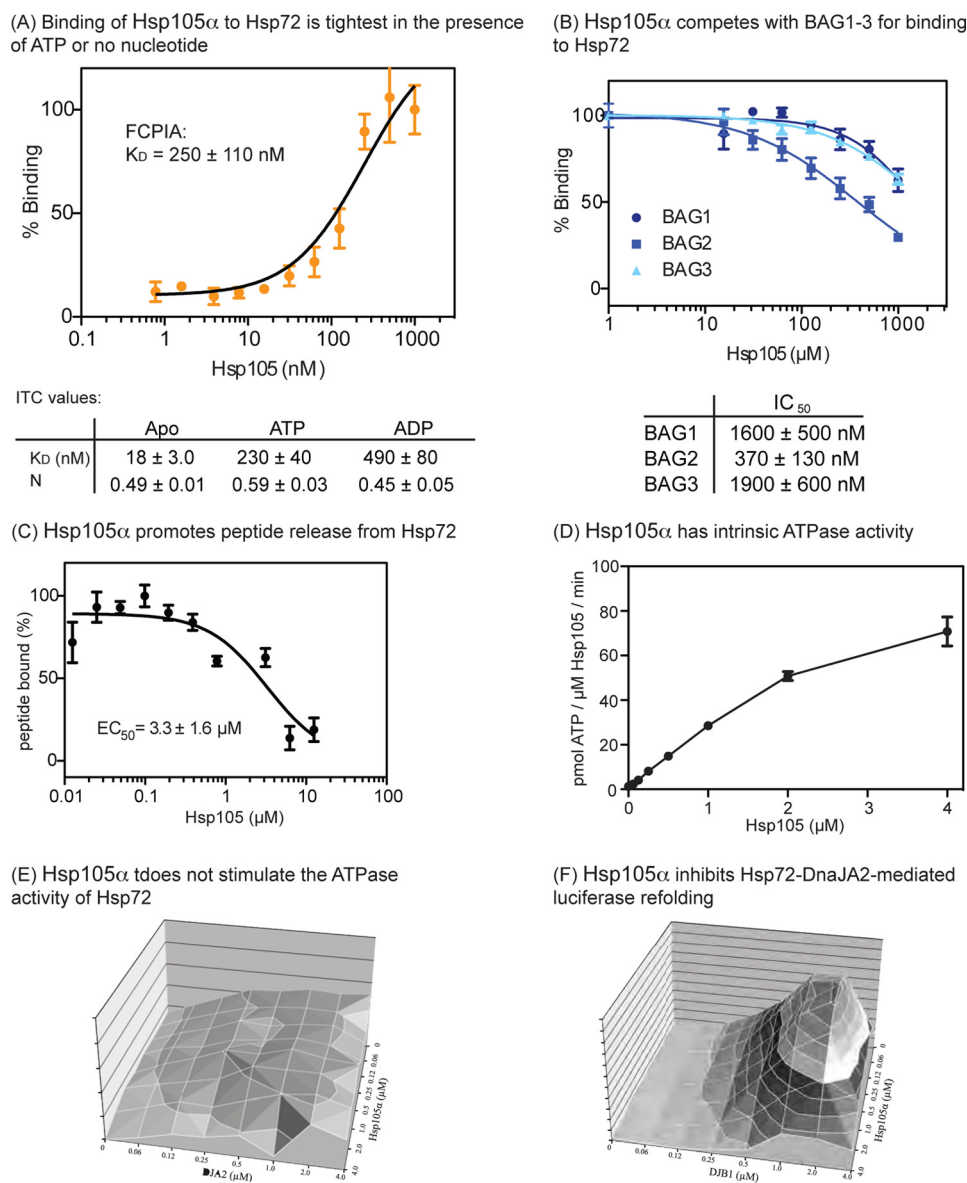


FIGURE 6. **Hsp105 α competes with BAG1–3 and acts as a NEF for Hsp72.** *A*, Alexa Fluor 488-labeled Hsp105 α binds to the immobilized ATP-bound form of Hsp72, as measured by FCPIA. The binding to apo-, ADP-, and ATP-bound Hsp72_{NBD} was confirmed by ITC (data not shown). *B*, Hsp105 competes with Alexa Fluor 647-labeled BAG1–3, as measured by FCPIA. *C*, Hsp105 promotes the release of HLA-FAM peptide from Hsp72, as measured by fluorescence polarization. *D*, Hsp105 has ATPase activity independently of Hsp72. This intrinsic activity was subtracted from subsequent ATPase studies. *E*, Hsp105 does not appear to strongly promote the ATPase activity of Hsp72. The y axis shows pmol ATP/ μ M Hsp72/min. For the full dataset, see [supplemental Fig. 3](#). *F*, Hsp105 strongly inhibits luciferase refolding by Hsp72 and J proteins. The y axis shows raw luminescence, with each tick representing 10,000 units. The error is \sim 5–10% of the value. For the full dataset, see [supplemental Fig. 3](#).

[supplemental Fig. 2](#)). In the presence of the correct J protein, BAG3 was a more potent stimulator of refolding than BAG1, whereas BAG2 was only inhibitory, regardless of the J protein partner (Fig. 5*B* and [supplemental Fig. 2](#)). When these experiments were repeated in the presence of 10 mM phosphate to suppress J protein activity, the activities of the BAG proteins were even more dramatic. For example, Bag3 now accelerated the folding activity of the Hsp72-DnaJB4 combination almost 10-fold (Fig. 5*B*). Notably, the ability of BAG2 to stimulate refolding was much more obvious in the presence of phosphate (Fig. 5*B*), showing that all of the BAG proteins that we tested can promote refolding under the right conditions. Together, these results (Fig. 5*C*) suggest that some combinations of Hsp72 and its cochaperones are competent for folding luciferase.

Hsp105 Competes with BAG Proteins and Acts as a NEF— Thus far, we have focused on the BAG family of NEFs because individual members of the family are linked to specific biological pathways, such as cell survival, the proteasome, and the autophagy system (32, 33, 35–40). The Hsp110 family is an evolutionarily distinct category of eukaryotic NEFs, and little is known about their biological roles. These proteins have a structure reminiscent of Hsp70, with an NBD and SBD (69). The NBD of Hsp110s binds nucleotide (70), and the SBD has an affinity for peptide substrates (71). However, members of the Hsp110 family lack the ability to refold clients (72, 73), and, rather, they have a prominent NEF function on Hsp70s (41, 74). Recently, human Hsp110 (termed Hsp105) has been shown to help coordinate stabilization of the cystic fibrosis conductance

receptor (75), suggesting that this NEF function might be functionally important. However, there is little known about the biochemistry of Hsp105 and its relative position in the hierarchy of eukaryotic NEFs. The structure of a yeast Hsp110 protein (Sse1p) with yeast Hsp70 (Ssa1) shows that the surface involved in the contact is partly overlapping with that used by the BAG proteins (76, 77). However, it has yet to be shown whether these NEFs compete. To better understand Hsp105 and compare it to the BAG family of NEFs, we used our battery of assays. In the FCPIA platform, Hsp105 α bound Hsp72 with an affinity of $\sim 250 \pm 110$ nM in the presence of ATP (Fig. 6A). Consistent with this value, Hsp105 bound ATP-Hsp72_{NBD} with an affinity of 230 ± 40 nM by ITC (Fig. 6A), suggesting that Hsp105 binds exclusively to the NBD. Similar to what we observed for BAG1–3, Hsp105 had a tighter affinity for the nucleotide-free Hsp72_{NBD} ($K_D = 18 \pm 3$ nM), and binding to the ADP-bound form was substantially weaker ($K_D = 490 \pm 80$ nM). The tight binding of Hsp105 to apo-Hsp72 was somewhat unexpected because binding between the yeast orthologs (Sse1 and Ssa1) has been shown to require nucleotide (78). However, there are functional differences between human and yeast Hsp105 orthologs (79), so their distinct preferences for nucleotide in Hsp72 might signify broader differences. Our ITC studies also suggest that Hsp105 might bind Hsp72 as a dimer because the N values were ~ 0.5 under all nucleotide conditions.

To test whether human Hsp105 could compete with BAG proteins, we immobilized Hsp72 on beads and measured the binding to labeled BAG proteins. In this FCPIA platform, Hsp105 competed for binding of Hsp72 to BAG1, BAG2, and BAG3 (Fig. 6B). Consistent with the hierarchy of binding affinities, Hsp105 was best able to compete for the weakest NEF-Hsp72 interaction (BAG2 IC₅₀, 370 ± 130 nM). Like the BAG proteins, Hsp105 accelerated the release of HLA-FAM (Fig. 6C), confirming that it is a *bona fide* NEF. However, Hsp105 had the intrinsic ability to bind ATP-FAM and hydrolyze ATP (Fig. 6D) (80), so its ability to promote nucleotide release could not be reliably tested. Finally, when we combined Hsp105 with Hsp72 and the four J proteins, we found that it was unable to significantly promote nucleotide hydrolysis of any of the Hsp72 combinations (Fig. 6E and supplemental Fig. 3), even after correcting for the intrinsic activity of Hsp105. Thus, it seems that Hsp105 accelerated client release without directly promoting ATPase activity. To test its effects in luciferase refolding experiments, we titrated Hsp105 into solutions of Hsp72 and either DnaJA2, DnaJB1, or DnaJB4. Hsp105 lacked intrinsic refolding activity, but it strongly inhibited Hsp72-mediated refolding (Fig. 6F and supplemental Fig. 3) by all three J proteins. These studies show that human Hsp105 is a NEF and that it combines with Hsp70 and its other cochaperones to expand the diversity of chaperone combinations.

DISCUSSION

In eukaryotes, the expansion of the number of Hsp70 cochaperones suggests that these proteins might have evolved specialized functions. Indeed, a number of studies in yeast and other models have supported this general concept. For example, the endoplasmic reticulum-resident Hsp70, BiP, works with a specific J protein (Sec63p) to coordinate translocation of

clients into the compartment, but it works with another J protein (Jem1p) to coordinate endoplasmic reticulum-associated degradation (81). Likewise, the J proteins Zuo1 and Jjj1 appear to be specialized for ribosome-associated client folding in yeast (22). Similar to what has been observed with J proteins, NEFs appear to be associated with guiding Hsp70 into specific functional roles. BAG1 is involved in multiple processes, including proteasomal degradation (32–34), whereas BAG3 is linked to autophagy (37), and BAG2 coordinates the removal of protein aggregates (36). These observations all suggest that Hsp70 might collaborate with (or “select”) specific cochaperones to extend its functionality in eukaryotes. A handful of studies using purified proteins have also supported the idea that cochaperones might differentially adjust biochemical properties *in vitro*. The clearest evidence comes from the Young group, in which human DnaJA1, but not the highly related DnaJA2, was found to work with Hsp70 to refold denatured luciferase (21, 58). On the basis of these results and our own, an intriguing hypothesis is that Hsp70 complexes might not only have distinct cellular functions, but that their biochemical properties might also differentiate them.

In this study, we first characterized how the BAG1–3 and Hsp105 proteins bound human Hsp72 *in vitro*. These studies revealed a strong hierarchy of binding, with BAG3 being the tightest partner and BAG2 being the weakest. BAG3 is the only stress-inducible BAG family member (82), so it is possible that this cochaperone might effectively outcompete other NEFs under certain cellular conditions. Conversely, BAG2 is the most abundant BAG protein in non-stressed HeLa cells (83), so its concentration might partially compensate for its weaker affinity. We found that Hsp105 competes with the BAG proteins for binding to Hsp70, suggesting that only one NEF (regardless to which class it belongs) can bind each molecule of Hsp70 at one time. Recent studies have shown that Hsp70-interacting protein (84), Hsp70 binding protein 1 (HSPBP1) (85), and some chemical Hsp70 inhibitors (86) also converge on this same region of the NBD, suggesting that this surface is a hub for protein-protein and protein-ligand interactions. Coevolution studies support this notion (67). Hsp70-interacting protein binds with an affinity of ~ 8 μ M by ITC (84), at least 100- to 1000-fold weaker than BAG3. It will be interesting to understand how the NEFs and other cochaperones might use secondary interactions (*e.g.* multivalent contacts with clients or other scaffolding proteins) to better compete for binding to the Hsp70_{NBD}, especially if they have relatively poor intrinsic affinities.

All of the NEFs we tested were able to accelerate the release of fluorescent peptide from Hsp72, and the BAG proteins promoted ATP-FAM release, suggesting that all of the human proteins are indeed NEFs *in vitro*. Further, all of the NEFs appeared to use a mechanism that involved stabilization of the nucleotide-free form of Hsp72. Thus, although the cocrystal structures suggested previously that they might help open the NBD and release ADP, our results show that all of the NEFs achieve this objective by strongly favoring the apo form of Hsp70. Given the high levels of ATP in the cytosol of most mammalian cells, it seems possible that NEF release might be mediated, in part, through rebinding of Hsp72 to this nucleotide and subsequent

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weakening of the NEF-Hsp72 interaction. Indeed, ATP and ADP were both able to displace BAG1–3 proteins from Hsp72, as measured by FCPIA. These results suggest an interplay between NEF binding and nucleotide status. Does the Hsp72-BAG complex ever exist in the nucleotide-bound state? Previous work using radiolabeled nucleotides clearly showed that stable BAG1-Hsc70 complexes are at least partially bound to ATP and ADP (53). Thus, cycling in the Hsp70 complex likely involves dynamic interactions between J proteins, NEFs, and nucleotides.

In most of our studies, the BAG domain appeared to be critical for interaction with Hsp72 and for mediating NEF activities. However, results with the BAG1C truncation suggested that peptide release, but not nucleotide release, may involve regions outside of the BAG domain. Consistent with this idea, full-length BAG1 also bound tighter than BAG1C. It is not currently clear how regions outside of the BAG domain might interact with Hsp70s or whether other NEFs share this feature.

To study the function of the reconstituted chaperone systems, we titrated Hsp72 with the four NEFs and the four major cytosolic J proteins to generate 16 different systems. Using ATP turnover and luciferase refolding as two representative chaperone activities, we found that the identity and stoichiometry of each component were important. Some systems, such as Hsp72 plus DnaJA1 and low levels of BAG3, were especially potent ATPase machines, whereas others, such as combinations of Hsp72 with DnaJA2 and high levels of BAG1, had negligible hydrolysis activity. All of the binary combinations of Hsp72 with J proteins had similar ATPase activity, so it appeared that the NEFs were the major determinant of differentiation. For example, the ATPase activity of Hsp72-DnaJA2 was activated by high levels of BAG2 and inhibited by BAG3 or Hsp105. However, it cannot be ignored that the identity of the J protein was important in combination with the NEF. For example, high levels of BAG3 were strongly inhibitory to Hsp72-DnaJB4 combinations but relatively less able to act on the Hsp72-DnaJA1 pair. Thus, it was the combination of the chaperone and both cochaperones that dictated the enzymatic activity of the system. This concept was even more dramatically exemplified by the results of the luciferase refolding studies. Although Hsp72 could refold luciferase in collaboration with DnaJA2, DnaJB1, and DnaJB4, the NEFs were all able to suppress this activity at high concentrations. BAG3 is stress-inducible, so we speculate that it might be advantageous for this protein to suppress costly refolding activity during conditions of stress. At lower concentrations of NEFs, even more interesting patterns emerged. For example, BAG1 and BAG3 could synergize with the Hsp72-DnaJB1 and Hsp72-DnaJB4 pairs but not the Hsp72-DnaJA2 combination. When physiological concentrations of phosphate were added to suppress the contribution of the J proteins, the NEF influence on refolding activity was even more exaggerated. Most striking, all three BAG proteins had a strongly stimulatory effect at low stoichiometry. These results clearly demonstrated that some permutations of Hsp72 and its cochaperones could fold luciferase, whereas others were less capable or inactive. Thus, some chaperone combinations can indeed be differentiated by their biochemical properties as well as their cellular functions.

It seems likely that the chaperone systems that we labeled as “inactive” are, instead, specialized for a biochemical activity that was poorly represented by our choice of *in vitro* assays. For example, none of the combinations that included Hsp105 were able to fold luciferase in our assays, suggesting that it may assist Hsp70 with other functions, such as cystic fibrosis conductance receptor trafficking and quality control (75). On the basis of this idea, it is intriguing to speculate that an impressive number of permutations might be generated by combinatorial assembly of human cochaperones. Moreover, some of these systems might have emergent biochemical properties that make them specialized for a subset of Hsp70 functions.

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