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Understanding the Functional Interplay between Mammalian Mitochondrial Hsp70 Chaperone Machine Components*[S]

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Mitochondria biogenesis requires the import of several precursor proteins that are synthesized in the cytosol. The mitochondrial heat shock protein 70 (mtHsp70) machinery components are highly conserved among eukaryotes, including humans. However, the functional properties of human mtHsp70 machinery components have not been characterized among all eukaryotic families. To study the functional interactions, we have reconstituted the components of the mtHsp70 chaperone machine (Hsp70/J-protein/GrpE/Hep) and systematically analyzed *in vitro* conditions for biochemical functions. We observed that the sequence-specific interaction of human mtHsp70 toward mitochondrial client proteins differs significantly from its yeast counterpart Ssc1. Interestingly, the helical lid of human mtHsp70 was found dispensable to the binding of P5 peptide as compared with the other Hsp70s. We observed that the two human mitochondrial matrix J-protein splice variants differentially regulate the mtHsp70 chaperone cycle. Strikingly, our results demonstrated that human Hsp70 escort protein (Hep) possesses a unique ability to stimulate the ATPase activity of mtHsp70 as well as to prevent the aggregation of unfolded client proteins similar to J-proteins. We observed that Hep binds with the C terminus of mtHsp70 in a full-length context and this interaction is distinctly different from unfolded client-specific or J-protein binding. In addition, we found that the interaction of Hep at the C terminus of mtHsp70 is regulated by the helical lid region. However, the interaction of Hep at the ATPase domain of the human mtHsp70 is mutually exclusive with J-proteins, thus promoting a similar conformational change that leads to ATPase stimulation. Additionally, we highlight the biochemical defects of the mtHsp70 mutant (G489E) associated with a myelodysplastic syndrome.

Mitochondria are ubiquitous, complex, and essential organelles of eukaryotes. Several biochemical reactions in iron metabolism, amino acid biosynthesis, urea metabolism, nucleotide biosynthesis, fatty acid metabolism, and oxidative phosphorylation are carried out within this organelle (1). More than 98% of the proteins that comprise mitochondria are encoded by the nuclear genome (2–4). Because a vast majority of proteins in the mitochondrial matrix are synthesized on cytosolic ribosomes, efficient import of proteins is critical for mitochondrial function (2, 3, 5, 6). This is assisted by mitochondrial chaperone machinery consisting of the 70-kDa family (mtHsp70),[2] J-proteins, and nucleotide exchange factors, which are critical for several cellular functions, including preprotein translocation and their subsequent folding inside the mitochondrial matrix (5, 7).

The mtHsp70 chaperone machinery components are highly conserved across species, including in mammalian mitochondria (8). The mtHsp70s are the most abundant and are usually present as one or two copies in the mammalian mitochondrial matrix compartment (9, 10). In the mammalian system, human mtHsp70 is commonly referred to as "*mortalin*" or glucose-regulated protein (GRP75) (11, 12). The mouse mitochondrion contains two isoforms of mtHsp70 termed as "mot-1" and "mot-2." Both isoforms are involved in several important cellular processes. In contrast to murines, human mitochondria have only one mtHsp70 encoded by the *HSPA9* gene, which functionally corresponds to mouse mot-2 (9, 10). Similarly, in human mitochondria, there are two members of type-I class Hsp40 isoforms reported, which are usually referred to as hTid-1_L (Larger isoform) and hTid-1_S (Smaller isoform). However, the physiological importance of these two J-proteins is still unclear (13). The different nucleotide states of human mtHsp70 are regulated by the nucleotide exchange factor: human GrpEL1 (14, 15). Similarly, a mammalian mitochondrion also possesses an ortholog of recently discovered "yeast Zim17" termed as hsp70 escort protein, "Hep" (16, 17). The physiological role of Hep in human mitochondrial matrix compartment is poorly understood. *In vivo*, the functional cycle of interaction of an unfolded mitochondrial client protein is initiated in the ATP-bound state of mtHsp70. The binding of the client protein in the peptide binding cleft and simultaneous interaction of J-domains of J-proteins with the ATPase domain of Hsp70 results in ATP hydrolysis, and thus stabilizes the interaction of a client polypeptide by converting Hsp70 to the ADP-bound state (18). Nucleotide release factors cause exchange of ADP for ATP, resulting in dissociation of bound clients, and prime Hsp70 for a second cycle of interaction (19–21).

In the mammalian system, the mtHsp70 machinery plays a very critical functional role for protein quality control in the

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[2] The abbreviations used are: mtHsp70, mitochondrial Hsp70; SBD, substrate binding domain; Hep, human escort protein; GST, glutathione *S*-transferase; MDS, myelodysplastic syndrome; GRP, glucose-regulated protein.

matrix compartment, thereby regulating mitochondria biogenesis. Importantly, the altered expression and specific mutations in the mtHsp70 chaperone machinery lead to severe mitochondrial disorders. In humans, the levels of mtHsp70 are highly up-regulated in all types of tumors and have been used as biomarkers to detect tumor invasiveness (22). Reduced expression of mtHsp70 is commonly observed in Parkinson disease and other age-related diseases leading to mitochondrial dysfunction (23). In zebrafish, a single point mutation in the C-terminal region of mtHsp70 (G492E, referred as MDS mutant) is associated with a hematopoietic defect similar to the myelodysplastic syndrome, presumably due to loss of chaperone function (24). Recently, it has been shown that altered expression of mammalian Hsp40 isoforms (hTid-1_L and hTid-1_S) leads to several cellular phenotypes, including apoptosis, malignancy, and cardiac-related disorders such as dilated cardiomyopathic syndrome (13, 25). Despite these critical cellular roles, little functional information at the biochemical level of human mtHsp70 chaperone machinery is available.

To address the functional importance of mammalian mtHsp70 chaperone machinery components in mitochondria biogenesis, we have undertaken the reconstitution of the human mtHsp70 machine to analyze its unique properties at the biochemical level as compared with the well explored model organism *Saccharomyces cerevisiae*. Our studies identified several novel biochemical features that are distinct from other Hsp70 systems and are critical for the mammalian mitochondrial function. Although mtHsp70 is highly conserved across species, we observed significant differences in sequence-specific binding toward mitochondrial client proteins and the mechanism of regulation through co-chaperones such as J-proteins. From our results, it is evident that such changes are mainly attributable to overall variations in the architecture of the substrate binding domain acquired during the evolution. Additionally, we have unraveled several novel properties of Hep, such as its unique nature to stimulate ATPase activity of mtHsp70 as well as prevention of aggregation of non-native unfolded proteins. By reconstitution analysis, we have uncovered several biochemical defects associated with the MDS mutant to understand the functional relevance of mtHsp70 machinery in disease conditions.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—Human *MOT2, DNAJA3* isoforms 1 and 2, and *GRPEL1* open reading frames were PCR-amplified from HeLa cells cDNA library (Stratagene) using sequence specific primers. For purification analysis, the His_6 tag was introduced at the N terminus of human mtHsp70, while at the C terminus for other proteins. For solubilization of human mtHsp70 in the bacterial system, its open reading frame was cloned into pRSFDuet-1 vector along with the yeast Zim17 (26). *DNAJA3* isoforms 1 and 2 and *GRPEL1* open reading frames were cloned into pET-3a vector. The human *HEP* clone (pOTB7) was obtained from Open Biosystems and subcloned into pET-3a vector. The GST fusion constructs of *HEP* and the substrate binding domain (SBD) of *HSPA9* were generated by introducing the respective coding sequences downstream of the GST tag in the pGEX-KG vector, respectively (27). Yeast Ssc1 was purified from *Escherichia coli* BL21(DE3) according to the procedure as previously described (28).

For generating deletion mutants of human mtHsp70, appropriate reverse primers were designed and cloned into the pRSFDuet-1 dual expression plasmid. Point mutants of human mtHsp70 and H/Q mutants of hTid-1_S were generated by QuikChange site-directed mutagenesis using high fidelity Pfu Turbo DNA Polymerase from Stratagene. All the clones were verified by DNA sequencing reactions carried out at Eurofins Inc. and Macrogen Inc. All the clones used for purification and analysis were devoid of mitochondrial leader sequence based on the reported mature forms and MITOPROT prediction software.

Expression and Purification of Human Mitochondrial Chaperone Machinery Components—For purification of His-tagged human mtHsp70, coexpression was carried out with yeast Zim17 in *E. coli* BL21(DE3) strain by allowing growth at 30 °C to an A_{600} of 0.6, followed by induction using 1 mM isopropyl 1-thio-β-D-galactopyranoside for 8 h. Human mtHsp70 was purified by standard affinity chromatography using nickel-nitrilotriacetic acid fast flow Sepharose. 500 ml of cell pellet was resuspended in 5 ml of lysis buffer A (25 mM HEPES-KOH, pH 7.5, 20 mM imidazole, 100 mM KCl, 10% glycerol) containing 2.5 mM magnesium acetate, 0.2 mg/ml of lysozyme and protease inhibitor mixture, followed by incubation at 4 °C for 1 h. The sample was gently lysed with 0.2% deoxycholate followed by DNase I (10 μg/ml) treatment for 15 min at 4 °C. The cell lysate was clarified by centrifuging at 28,000 $\times$ *g* for 30 min at 4 °C. The soluble supernatant was incubated with 500 μl of nickel-nitrilotriacetic acid-Sepharose (bed volume) for 2 h at 4 °C. Unbound proteins were removed by extensive washing with buffer A, followed by an additional wash with buffer A containing 0.5% Triton X-100. To remove nonspecific contaminants, the resin was extensively washed again with buffer B (25 mM HEPES-KOH, pH 7.5, 20 mM imidazole, 100 mM KCl, 10 mM magnesium acetate, 10% glycerol, and 2 mM ATP) at 4 °C, followed by 2 washes with high salt buffer C (25 mM HEPES-KOH, pH 7.5, 20 mM imidazole, 1 M KCl, 10 mM magnesium acetate, 10% glycerol). Nonspecific impurities were further removed by buffer A wash containing 40 mM imidazole. The bound proteins were eluted with buffer D (25 mM HEPES-KOH, pH 7.5, 250 mM imidazole, 100 mM KCl, 10 mM magnesium acetate, 10% glycerol) and the samples were dialyzed against appropriate buffers for use in particular experiments. All mtHsp70 deletion and point mutants were purified similar to wild type protein, unless otherwise specified.

Histidine-tagged hTid-1_L and hTid-1_S proteins were purified from the insoluble pellet fraction obtained by expressing them in the BL21(DE3) *dnaKJ*$^-$ *E. coli* strain and purified using a similar protocol as previously described for yeast Mdj1 (29). GrpEL1 and human Hep purification were done using similar procedures as described (29, 30). The full-length GST-Hep and GST alone proteins were purified according to the published protocols with minor modifications (28). The GST tag from the SBD of human mtHsp70 was cleaved by thrombin treatment according to the manufacturer's instructions (Novagen). Greater than 95% purity was obtained for the preparations of human mtHsp70 and its mutants, hTid-1_L, hTid-1_S, human

GrpEL1, and Hep as analyzed on SDS-PAGE (supplemental Fig. S1, *A* and *B*), size exclusion chromatography, and mass spectrometry (data not shown).

Fluorescence Anisotropy Peptide Binding Assays—25 nM Fluorescein-labeled P5 peptide (CALLLSAPRR) was incubated with increasing concentrations of wild type, deletions, MDS mutant of human mtHsp70, and yeast Ssc1 at 25 °C in buffer (25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10 mM magnesium acetate, 10% glycerol). After binding reached equilibrium, anisotropy measurements were recorded with the Beacon 2000 fluorescence polarization system (Invitrogen Corp.) at 25 °C with excitation at 490 nm and emission at 535 nm. The data were fitted to a quadratic single-site binding equation using Prism 4 (GraphPad) to calculate the equilibrium dissociation constant (K_d). Similarly, peptide corresponding to the presequence of cytochome *c* oxidase (Cox4-MLSLRQSIRFFKPTRRLC) was also used for binding measurements. For calculating k_{off} rates, excess unlabeled P5 peptide was added and measurements were recorded. The values were fitted to a one-phase exponential dissociation equation using Prism 4.0.

Single Turnover ATPase Assays—Human mtHsp70-ATP complexes were prepared according to the procedure as previously described (31). Briefly, 100 μg of mtHsp70 protein (wild type, deletion, and MDS mutant) was incubated with 50 μCi of [α-^{32}P]ATP (BRIT, 10 mCi/ml) in 100 μl of buffer X (25 mM HEPES-KOH, pH 7.5, 100 mM KCl, and 10 mM magnesium acetate) on ice for 3 min. The complex was immediately isolated on a NICK column (GE Healthcare). Aliquots of 1 μM mtHsp70-[α-^{32}P]ATP complexes containing 10% glycerol were frozen in liquid nitrogen and stored at −80 °C. Single turnover experiments were performed in buffer A at 25 °C in the presence of various concentrations of proteins (hTid-1$_S$, hTid-1$_L$, Hep, and GrpEL1) and P5 peptide as previously described (32). The reaction was stopped at various time intervals and the mixture was separated on thin layer chromatography plates (TLC) and exposed to phosphorimager cassettes (33). The percent conversion of ATP to ADP was determined and the rate of ATP hydrolysis was fitted to a first-order rate equation by nonlinear regression analysis using Prism 4.0.

Rhodanese Aggregation Assays—Bovine liver rhodanese (Sigma) was used as a model substrate for analyzing the aggregation prevention activity of human mtHsp70 machine components using the same procedure as previously described (34). The prevention of rhodanese aggregation by each specific chaperone (mtHsp70, hTid-1$_S$, hTid-1$_L$, and Hep) was analyzed independently or in combination using the appropriate buffer as described (34). The percentage of aggregation was calculated by setting the total value in the absence of chaperones as 100%.

In Vitro GST Pulldown Analysis—Purified GST-Hep (1 μM) was incubated with a 10-μl bed volume of glutathione-agarose beads in 150 μl of GST buffer (20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 10 mM magnesium acetate, 0.2% Triton X-100). After washing to remove unbound proteins, the beads were blocked with 0.1% bovine serum albumin for 20 min at 23 °C. The beads were washed 2 times in GST buffer to remove excess unbound bovine serum albumin. The GST-bound beads were then resuspended in 200 μl of binding buffer and incubated with 2 μM human mtHsp70 (wild type, deletions, and SBD mutants) for 30 min at 23 °C. The incubation was continued for an additional 5 min with the addition of 2 mM ATP or ADP in the same reaction mixture. After washing the beads 3 times in GST buffer, bound proteins were resolved on SDS-PAGE followed by Coomassie dye staining.

RESULTS

Conserved Hsp70 Shows a Difference in Sequence Specificity Across the Species—Yeast mitochondrial matrix has three members of Hsp70 (Ssc1, Ssq1, and Ecm10) dedicated to several specialized functions (35). To the present date, our major understanding regarding functional interplay between various matrix chaperone components is derived primarily from studies conducted in yeast. In contrast, higher eukaryotic systems such as human mitochondria contain only one mtHsp70 dedicated to all diverse cellular functions. The unique biochemical features acquired by human mtHsp70 during higher eukaryotic evolution that is critical to its myriad cellular functions have not been investigated. Therefore, to understand the molecular interactions between human mtHsp70 chaperone components, we have attempted for the first time to purify major components of the human mtHsp70 chaperone machine and have functionally reconstituted them *in vitro*.

To explore the functional versatility of human mtHsp70 at the biochemical level, we have analyzed several parameters of the human mitochondrial Hsp70/J-protein/GrpE/Hep system comparing them with yeast Ssc1. As one of the parameters, the difference in mitochondrial client protein binding properties between human mtHsp70 and yeast Ssc1 was investigated. The client protein binding affinities of human mtHsp70 and yeast Ssc1 were analyzed using a fluorescence anisotropy-based peptide binding assay. We have utilized two model mitochondrial targeting sequence-derived peptides; 1) P5 peptide (CALLLSAPRR), having a portion of the mitochondrial targeting sequence of aspartate aminotransferase from chicken and 2) a portion of the yeast cytochrome oxidase 4 mitochondrial targeting sequence peptide (MLSLRQSIRFFKPTRRLC) named Cox4. Both peptides were labeled with fluorescein fluorophore covalently attached to the cysteine residue. The underlying principle behind the fluorescence anisotropy assay is a change in the relative tumbling rates of the fluorescent labeled peptides in free and bound forms of Hsp70 in solution. The kinetic parameters obtained are used for measurements of relative affinities for the client proteins in different nucleotide bound states of mtHsp70.

Human mtHsp70 yielded a very high dissociation constant (K_d) of 14.9 μM for the P5 peptide (Fig. 1*A* and supplemental Table S1). Interestingly, the affinity of human mtHsp70 for P5 peptide was 50-fold lower than its yeast ortholog: Ssc1 (K_d of 0.3 μM) (Fig. 1*A* and supplemental Table S1). To further assess the difference in the affinity for P5 peptide between yeast Ssc1 and human mtHsp70, we have measured the release rate (k_{off}) of bound labeled P5 peptide complex upon addition of an excess of unlabeled P5 peptide. Human mtHsp70 revealed ~2.7-fold greater k_{off} of 0.41 (±0.007) min^{-1} for P5 peptide as compared to yeast Ssc1, suggesting that the inherent lower affinity of wild type human mtHsp70 toward the P5 peptide is likely due to

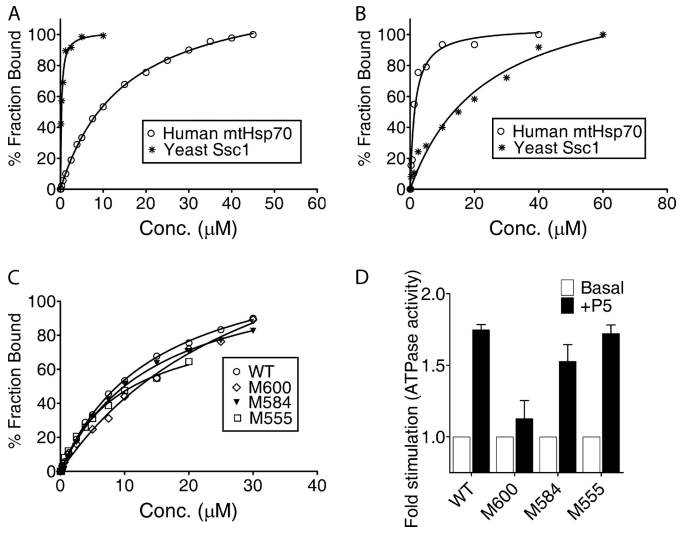

FIGURE 1. **Fluorescence anisotropy assay of peptide binding for yeast Ssc1, wild type, and C-terminal deletion mutants of human mtHsp70.** *A* and *B*, 25 nM fluorescein-labeled P5 (*F-P5*) and Cox4 (*F-Cox4*) peptide were incubated in the presence of the indicated concentrations of purified yeast Ssc1 and human mtHsp70. Fluorescence anisotropy measurements were taken as described under "Experimental Procedures." *C*, 25 nM F-P5 peptide was incubated in the presence of the indicated concentrations of wild type (*WT*) and C-terminal deletion mutants (*M600*, *M584*, and *M555*) of human mtHsp70. After binding reached equilibrium, the anisotropy values were recorded at 25 °C. The normalized values were plotted as a function of increasing concentrations of different proteins as indicated. *D*, 1 μM radiolabeled ATP complex of wild type and deletion mutants (*M600*, *M584*, and *M555*) of human mtHsp70 were prepared and incubated with 100 μM P5 substrate. ATP hydrolysis was monitored as a function of time at 25 °C. The percentage of ATP to ADP conversion at various time intervals was plotted and fitted into one-phase exponential association binding analysis using Prism 4 to calculate the hydrolysis rate constant. Fold-stimulation was calculated by setting the intrinsic ATP hydrolysis rate as 1. *Error bars* are derived from two independent sets of experiments.

enhanced release and on rate for peptide binding (supplemental Table S1).

To identify the client specificity of human mtHsp70 and its relative affinities for different peptide substrates, we have also performed peptide binding analysis using the larger (18-mer) hydrophobic Cox4 peptide. Surprisingly, wild type human mtHsp70 showed a higher affinity with K_d of 1.49 μM for Cox4 peptide in the ADP-bound state (Fig. 1*B* and supplemental Table S1). Thus, human mtHsp70 showed 10-fold greater affinity toward larger peptides that contain more hydrophobic sequences, such as Cox4 as in comparison to conventional P5 peptide. However, yeast Ssc1 showed an affinity with a K_d of 23.6 μM for Cox4 peptide that is 15.8-fold lower compared to human mtHsp70 (Fig. 1*B* and supplemental Table S1). Interestingly, this affinity of yeast Ssc1 for Cox4 peptide was found significantly lower (~78-fold) than its affinity toward the P5 peptide (Fig. 1*B* and supplemental Table S1). These observations suggest that, although the functional specificity of mtHsp70 across species is highly conserved, significant differences exist at the biochemical level with respect to their affinities toward the client protein interaction.

C-terminal 10-kDa Helical Lid of Human mtHsp70 Is Dispensable for Smaller P5 Peptide Interaction—The deletion of the complete C-terminal helical lid in yeast Ssc1 resulted in a lethal phenotype (36). Also, the lidless variant of yeast Ssc1 showed a severely reduced affinity for peptide binding (36). To probe the potential role of the helical lid in the observed peptide binding affinities of human mtHsp70, we generated 3 C-terminal deletion mutants by removing C to E helices (M600), truncating at the middle of helix B (M584), and deleting the entire helical region (M555) (supplemental Fig. S1, *B* and *C*). The purified deletion mutants of mtHsp70 were assessed for the peptide interaction utilizing the fluorescence anisotropic measurements using labeled P5. In contrast to yeast Ssc1, the dissociation equilibrium constants for the C-terminal human mtHsp70 truncations (M600, M584, and M555) were not significantly altered except that, the M600 mutant displayed a 2-fold lower affinity for P5 binding (Fig. 1*C* and supplemental Table S2). We speculate that, a less drastic influence of the helical lid on the P5 peptide interaction is either due to an overall differential helical-fold of variable domain or the altered orientation of the helical lid over the peptide binding cleft, as observed in rat Hsc70 (37, 38). Additionally, higher k_{off} observed for the wild type protein also further supports the idea that the helical lid region of human mtHsp70 is not involved in regulating the half-life of the substrate complex in the ADP-bound state, possibly due to a partial open conformation of SBD.

Binding of client proteins to SBD brings a global conformational change in the ATPase domain, thus enhancing the rate of ATP hydrolysis of Hsp70 (39). To analyze the effect of the helical lid domain in the interdomain communication, we tested the ability of deletion mutants to stimulate its ATPase activity upon P5 binding using single turnover experiments. We observed that fold-stimulation of the ATPase activity of the deletion mutants correlated well with their peptide affinities, thus retaining a normal level of interdomain communication in deletion mutants (Fig. 1*D*). Similarly, the rate of ATP-dependent substrate release was found similar to wild type for the deletion mutants as observed by using fluorescence anisotropy measurement analysis (data not shown). In contrast, a partial loss in interdomain communication was observed for yeast Ssc1. These observations further support that the role of a helical lid with respect to interdomain communication is still dispensable for human mtHsp70. Taken together, we conclude that the C-terminal 10-kDa helical lid region of human mtHsp70 does not play a critical role in determining the affinities for P5 peptide binding as compared with other well explored Hsp70 systems.

Two Human Mitochondrial J-protein Isoforms, hTid-1$_L$ and hTid-1$_S$ and Hep Differentially Regulate the ATPase Activity of Human mtHsp70—As a second functional parameter, we have investigated the mechanism of interaction between human mtHsp70 with its multiple co-chaperones that are critical for regulation of the chaperone cycle in mitochondria biogenesis. The functional Hsp70 cycle is initiated in the ATP-bound form, followed by ATP hydrolysis stimulated by the concerted action of J-proteins and locking up the client proteins in the ADP state (18, 40, 41). Interestingly, the human mitochondrial matrix consists of two members of type-I DnaJ homologs namely, hTid-1$_L$ and hTid-1$_S$ in comparison to well studied yeast mitochondria. Human Tid-1$_S$ is a truncated splice variant of hTid-1$_L$ with an insertion of 6 new amino acids but lacking 33 amino acids of the larger isoform at the C terminus (13). To assess the

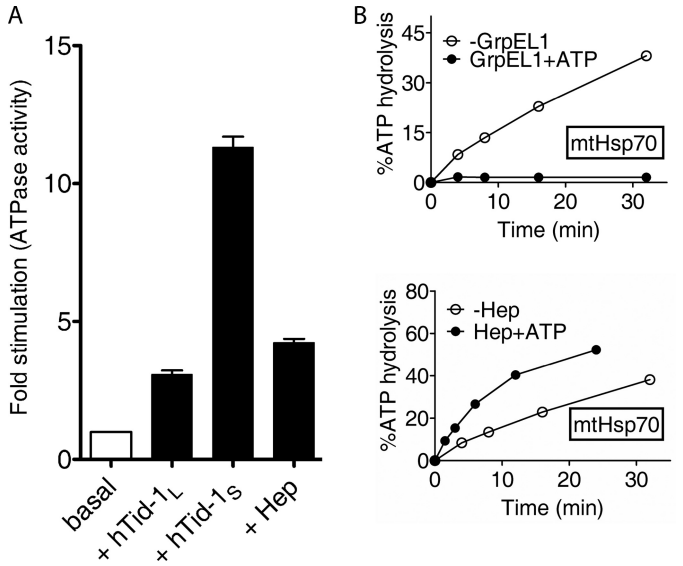

FIGURE 2. **Effect of J-proteins, Hep, and GrpEL1 on the ATPase activity of human mtHsp70.** The preformed radiolabeled mtHsp70-ATP complex (1 μM) was incubated with either (*A*) 1 μM hTid-1_L, or 1 μM hTid-1_S and 4 μM Hep. ATP hydrolysis was monitored under single turnover conditions at different time intervals. *B,* human mtHsp70-ATP complex (1 μM) was incubated with either buffer alone control (*open circles*) and 1 μM GrpEL1 (*upper panel*) or 2 μM Hep (*lower panel*) together with 250 μM unlabeled ATP (*closed circles*) at 25 °C. ATP hydrolysis was monitored as a function of time, and percentage of ATP to ADP conversion at various time intervals was plotted. Fold-stimulation was calculated by setting the intrinsic ATP hydrolysis rate as 1. *Error bars* are derived from two independent sets of experiments.

functional interaction of matrix J-proteins with wild type mtHsp70 protein, we again utilized well established single turnover ATPase experiments. The wild type human mtHsp70 had a basal rate constant of 0.032 min^{-1}. However, this basal rate is 2-fold slower than that of yeast Ssc1 and *E. coli* DnaK (42). To address the functional differences between these two matrix J-protein splice variants; their basic property of stimulating ATPase activity of human mtHsp70 was analyzed. At 1:1 molar ratio of mtHsp70 to J-protein, a 3.2-fold stimulation was observed with hTid-1_L for wild type protein (Fig. 2*A*). Interestingly, under similar conditions hTid-1_S showed an 11-fold robust stimulation for wild type mtHsp70 (Fig. 2*A*). Based on these results, it is tempting to speculate that the difference in the stimulatory activities among hTid-1 J-variants might contribute to their *in vivo* phenotypic differences as reported (13).

Hep belongs to a newly discovered class of zinc-binding proteins that have been implicated for maintaining the functional status of mitochondrial Hsp70 by actively modulating the conformations in different nucleotide bound states. The function of Hep1/Zim17 was found to be essential in yeast mitochondria. A similar ortholog exists in mammalian mitochondria including human, with a predicted analogous function. The function of Hep is least studied among all other co-chaperones and how Hep modulates the conformations of Hsp70s is still elusive. Previously, it was speculated that Hep may interact with human mtHsp70 and function as a nucleotide exchange factor (26). The nucleotide exchange factors are critical components of chaperone machinery that accelerates the rate of exchange between ADP to ATP by several orders of magnitude in a typical folding reaction. GrpEL1 is the proposed nucleotide exchange factor of the human mtHsp70 machine similar to yeast Mge1 and *E. coli* GrpE. To assess the nucleotide release activity of human GrpEL1, we used the single turnover ATPase assay to monitor the hydrolysis of the prebound mtHsp70-ATP complex of the wild type protein in the presence of GrpEL1. As indicated in Fig. 2*B, upper panel*, human GrpEL1 showed robust nucleotide exchange activity in the presence of excess unlabeled ATP, thus inhibiting the maximum ATP hydrolysis of wild type mtHsp70. Our results establish the true nature of the function of GrpEL1 as a nucleotide exchange factor of human mtHsp70. In contrast, even in the presence of an excess of unlabeled ATP, Hep retained the ability to stimulate the ATPase activity of human mtHsp70 (Fig. 2*B, lower panel*). Surprisingly, in contrast to its yeast Zim17, human Hep showed a significant ability to stimulate the ATP hydrolysis of mtHsp70 under single turnover conditions. At a 1:4 molar ratio of mtHsp70 to Hep, respectively, a 4.3-fold stimulation was obtained for wild type mtHsp70 (Fig. 2*A*). A significant increase in fold-stimulation was observed at higher concentrations of Hep (data not shown). In conclusion, based on the ability of Hep to stimulate the ATPase activity of mtHsp70 in comparison to GrpEL1 convincingly rules out speculation of Hep acting as a nucleotide exchange factor.

Nature of Hep Interaction with mtHsp70 Is Distinctly Different from a Substrate-specific Interaction—The unique ability of Hep to stimulate the ATPase activity in contrast to yeast Zim17 raises two intriguing questions. First, the interaction between Hep and mtHsp70 is substrate-specific or second, it functions similar to the canonical matrix J-proteins. To gain further insights into the true functional nature of Hep in the human mtHsp70 chaperone machine, we have analyzed the physical interaction between Hep and mtHsp70 in different nucleotide states using GST pulldown analysis. To investigate the stability of the interaction, we have briefly incubated the preformed GST-bound Hep-mtHsp70 complex in the presence or absence of nucleotides (ATP/ADP). As shown in Fig. 3*A, lane 1*, a stronger interaction was observed between Hep and mtHsp70 in the absence of nucleotides. However, stability of the complex was reduced 2-fold in the presence of ATP or ADP indicating that the affinity is compromised in nucleotide bound states of mtHsp70 (Fig. 3*A*, *lanes 2* and *3*). As a control, GST alone did not interact with wild type mtHsp70 (Fig. 3*A, lanes 4–6*).

To address the specificity of interaction between Hep and mtHsp70, the preformed complex was incubated in the presence of excess peptide substrates with different nucleotide bound forms of mtHsp70. The P5 peptide did not affect the stability of the Hep-mtHsp70 complex in the presence or absence of bound nucleotides (Fig. 3*B*, compare *lanes 1–2* with *3–4*). Our results confirm that the nature of interaction between mtHsp70 and Hep is not a Hsp70-substrate interaction. Recently, it was shown that Hep stably interacts with the isolated ATPase domain of human mtHsp70 by a gel filtration analysis (26). To address whether the substrate binding domain of human mtHsp70 can independently interact with Hep *in vitro*, we incubated various concentrations of His-tagged SBD of mtHsp70 with GST-Hep and subjected to pulldown analysis. No detectable levels of interaction between SBD and Hep were observed (Fig. 3*C, lanes 1–4*). However, these results did not

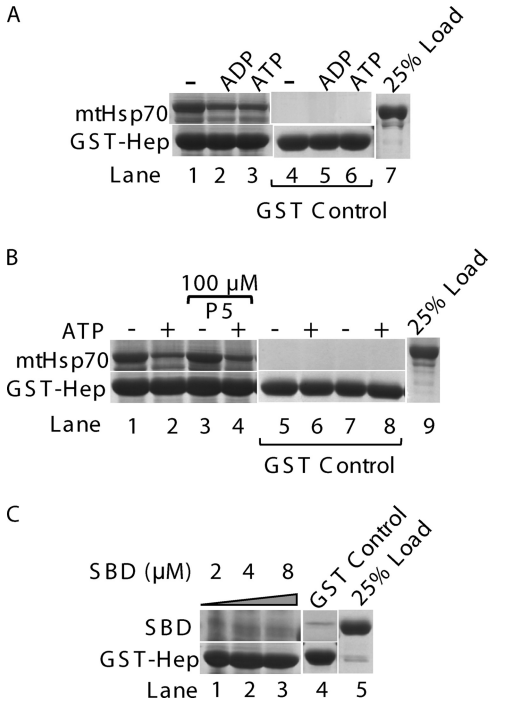

FIGURE 3. **Understanding the nature of Hep interaction with human mtHsp70.** *A,* immobilized GST-Hep (1 μM) was incubated with 2 μM His-tagged full-length mtHsp70 in the presence or absence of nucleotides (ATP/ADP). *B,* GST-Hep bound mtHsp70 complex was incubated with 100 μM P5 substrate in the presence or absence of ATP. *C,* 1 μM immobilized GST-Hep was incubated with increasing concentrations of SBD of mtHsp70 as indicated. The bound proteins were analyzed by SDS-PAGE followed by Coomassie dye staining. GST alone was used as a negative control and 25% input (offered to the beads) was used as a loading control.

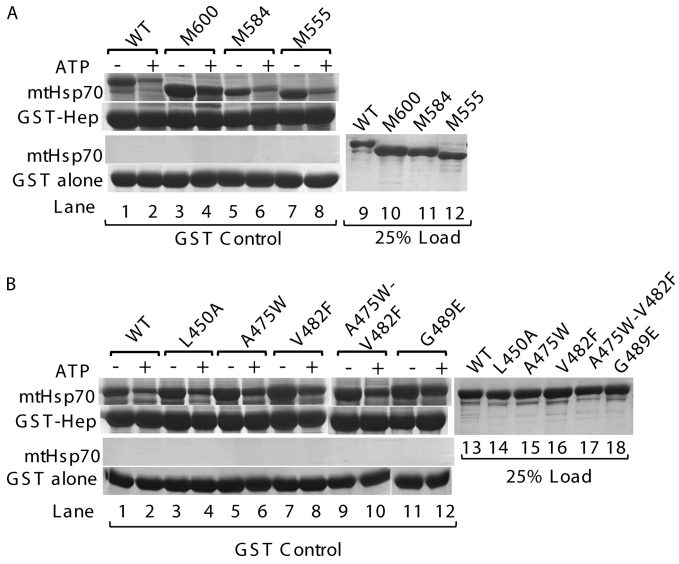

FIGURE 4. **Nucleotide-dependent Hep interaction analysis with SBD mutants of human mtHsp70.** Immobilized GST-Hep (1 μM) was incubated with: *A,* 2 μM wild type (WT), C-terminal deletions (*M600*, *M584*, and *M555*), and *B,* SBD mutants (L450A, A475W, V482F, A475W/V482F, and G489E) in the presence or absence of ATP. The bound proteins were analyzed by SDS-PAGE followed by Coomassie dye staining. GST alone was used as a negative control and 25% input of wild type human mtHsp70 and mutants was used as a loading control.

rule out the possibility of interaction between Hep and SBD of mtHsp70 in a full-length context.

Hep Binds to SBD of mtHsp70 in a Full-length Context and Its Interaction at the C Terminus Is Different from J-proteins—To analyze the Hep interaction in greater detail in a full-length context, we subjected deletion mutants of mtHsp70 for GST pulldown analysis using similar experimental conditions in different nucleotide bound states. Surprisingly, greater than 2-fold enhanced interaction was observed for the M600 deletion mutant in the non-nucleotide and ATP-bound states (Fig. 4*A*, compare *top two rows, lanes 1–2* and *3–4*). Interestingly, the interaction was restored to wild type levels in M584 and M555 deletion mutants (Fig. 4*A*, compare *top two rows, lanes 1–2* and *5–8*). As a control, all deletion mutants did not show any detectable levels of interaction with GST alone at similar concentrations utilized for monitoring the Hep interaction (Fig. 4*A*, *bottom two rows, lanes 1–8*). The results of this study clearly highlight the importance of the helical lid region in regulating the Hep interaction with SBD.

To test the influence of the peptide binding β-sandwich region in Hep interaction, we generated additional site-specific point mutants of mtHsp70 in the well conserved SBD pocket. These include arch mutants (L450A, A475W), the hydrophobic pocket mutant (V482F), and a double mutant that is a combination of arch and the hydrophobic pocket mutant (A475W/V482F) (supplemental Fig. S1, *B* and *C*). To validate their functional properties, we purified the mutant proteins and subjected them to peptide binding and J-protein stimulation analysis. Similar to *E. coli* DnaK, these mutants were found defective in peptide binding and failed to show stimulation by J-proteins (supplemental Fig. S2). Importantly, these mutants were tested for their ability to interact with Hep, based on GST pulldown analysis. Interestingly, all the mutants showed significant and enhanced interaction with Hep in non-nucleotide as well as in the ATP-bound state in comparison to wild type protein (Fig. 4*B, top two rows*, and compare *lanes 1–12*). As a control, GST alone did not show binding of any detectable trace amounts of mtHsp70 mutants (Fig. 4*B, bottom two rows, lanes 1–12*). Based on these findings, we propose that Hep interacts with both domains of the human mtHsp70 in a full-length context and the C-terminal helical lid is involved in regulating its interaction with SBD.

Type-I J-proteins are known to interact with SBD of Hsp70s in a full-length context. Based on biochemical and genetic data it has been speculated that J-protein interaction with SBD in the full-length context is similar to a typical substrate (42). Therefore, to differentiate Hep binding from J-protein interaction at the C terminus of mtHsp70, we compared their specific ATPase stimulating activities in deletion and point mutants. Interestingly, all mutants significantly retained Hep-dependent stimulating activity in contrast to the matrix J-proteins (hTid-1_L and hTid-1_S) stimulation (Fig. 5*A* and supplemental Fig. S2, *D* and *E*). These novel findings further strengthen our understanding that the Hep interaction at the C terminus of wild type mtHsp70 is not substrate specific and the interaction sites are not mutually exclusive with J-protein.

Binding Sites of Hep and J-proteins with the ATPase Domain of Human mtHsp70 Are Mutually Exclusive—Hep and J-proteins are known to interact with the ATPase domain of mtHsp70 and stimulate its ATPase activity (26, 43). To test whether the binding sites of Hep and J-domain of the J-proteins

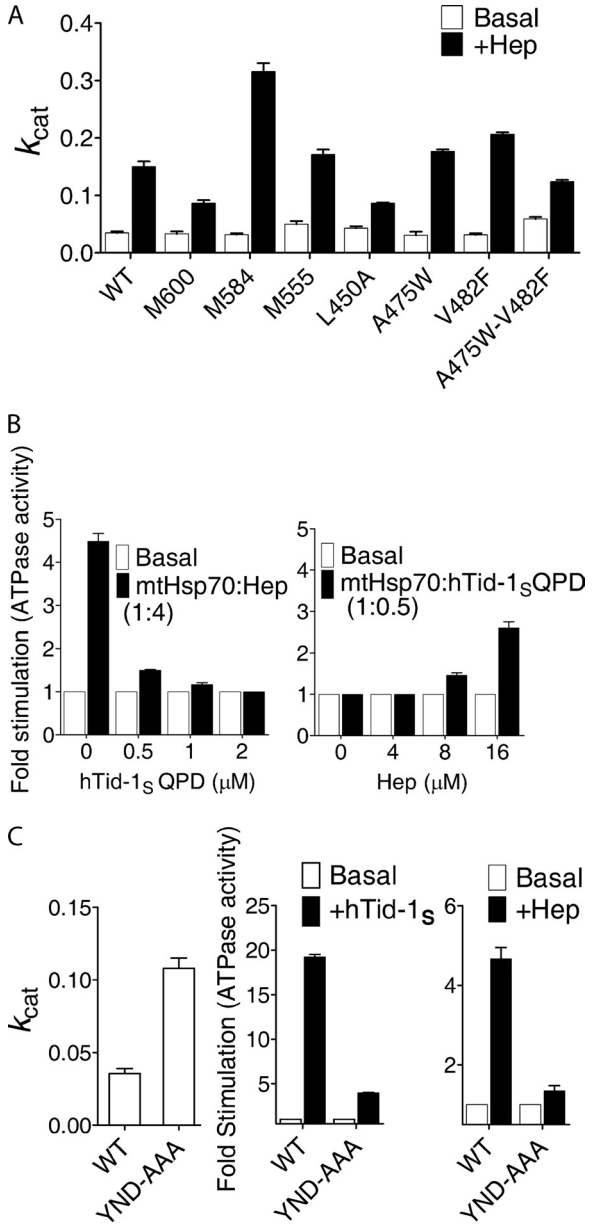

FIGURE 5. **Identification of Hep interaction sites at the SBD and ATPase domains of human mtHsp70.** *A*, the preformed 1 μM radiolabeled ATP complexes of wild type (*WT*), C-terminal deletions (*M600*, *M584*, and *M555*), and SBD mutants (L450A, A475W, V482F, and A475W/V482F) of mtHsp70 were incubated with 4 μM Hep and ATP hydrolysis was monitored as a function of time at 25 °C. *B*, a reaction mixture containing 1 μM radiolabeled human mtHsp70-ATP complex and 4 μM Hep (1:4 ratio) was incubated with increasing concentrations of hTid-1$_S$ QPD mutant (*left panel*). Similarly, a reaction mixture containing 1 μM radiolabeled human mtHsp70-ATP complex and 0.5 μM hTid-1$_S$ QPD mutant was incubated with increasing concentrations of Hep (*right panel*). *C*, 1 μM radiolabeled ATP complexes of wild type and mtHsp70 mutant (YND-AAA) was incubated with buffer alone (*left panel*), 2 μM hTid-1$_S$ (*middle panel*), and 4 μM Hep (*right panel*). ATP hydrolysis was monitored under single turnover conditions. Fold-stimulation was calculated by setting the intrinsic ATP hydrolysis rate as 1. *Error bars* are derived from two independent sets of experiments.

on the ATPase domain of mtHsp70 are similar, we performed competition experiments using single turnover ATPase assays. For this experiment, we utilized a mutant form of hTid-1$_S$ where histidine from the J-domain "HPD" signature sequence was replaced by glutamine (QPD). Previously, it was reported for J-proteins that the H/Q mutation in the J-domain abolishes its ability to stimulate ATPase activity despite binding to Hsp70 in the ATP-bound state (43). Similarly, we observed that the hTid-1$_S$ QPD mutant failed to stimulate the ATPase activity of human mtHsp70 even at higher concentrations (data not shown). To identify the binding sites, different molar concentrations of the hTid-1$_S$ QPD mutant were titrated against a constant ratio (4:1) of Hep to the mtHsp70-ATP complex. At a 4:1 ratio, Hep alone showed a 4.3-fold stimulatory activity against the preformed mtHsp70-ATP complex (Fig. 5*B, left panel, first pair of bars*). Interestingly, increasing concentrations of the hTid-1$_S$ QPD mutant showed a robust decline in the ability of Hep to stimulate the ATPase activity of mtHsp70 (Fig. 5*B, left panel, second to fourth pair of bars*). Conversely, a fixed ratio of hTid-1$_S$ QPD to the mtHsp70-ATP complex was competed by increasing concentrations of Hep, thus showing enhancement in ATPase stimulation of mtHsp70 (Fig. *5B, right panel,* compare the *first* and *second bars* to the rest). These novel findings suggested that Hep and J-protein interaction at the ATPase domain of human mtHsp70 is mutually exclusive or brings similar conformational changes upon binding, thus stimulating the ATPase activity of mtHsp70.

To identify the critical residues important for Hep interaction at the ATPase domain of human mtHsp70, we created a triple substitution mutant in the ATPase domain of mtHsp70 by replacing amino acids at positions 196, 198, and 199 to alanines (YND to AAA). The human mtHsp70 YND mutant showed 3-fold elevated basal activity in comparison to wild type (Fig. 5*C, first panel*). A similar mutation at corresponding amino acid sequences in *E. coli* DnaK resulted in decreased ATPase stimulation by J-proteins. Similarly, the YND mutant showed decreased stimulation by hTid-1$_S$ in comparison to wild type (Fig. 5*C, second panel*). Interestingly, the YND mtHsp70 mutant also showed a significant decrease in Hep stimulation as in comparison to the wild type protein further supporting the mutually exclusive nature of Hep and J-protein interaction at the ATPase domain of human mtHsp70 (Fig. 5*C, third panel*).

Human Mitochondrial Chaperone Machinery Components Prevent Aggregation of Unfolded Client Proteins—As a third functional parameter, we assessed the ability of matrix chaperone machine components in preventing the aggregation of client proteins. Molecular chaperones are known to prevent aggregation of client proteins in response to various types of physiological stress and play a critical role in maintenance of matrix protein quality control. Different members of the Hsp70 family have been shown to prevent aggregation to various extents depending on the nature of the substrate as well as robustness of the chaperone machinery. To understand this function, we monitored the *in vitro* aggregation of rhodanese as a model client protein. As shown in Fig. 6*A*, at 10-fold molar excess of human mtHsp70, ~70% protection was observed with denatured rhodanese. At similar molar ratios, bovine serum albumin as a control did not protect the denatured rhodanese against aggregation indicating that human mtHsp70 can specifically interact with an exposed hydrophobic core of the substrate to prevent formation of non-native conformations (Fig. 6*A*). Greater than 80% protection against aggregation was observed with higher molar ratios (1:20) of human mtHsp70

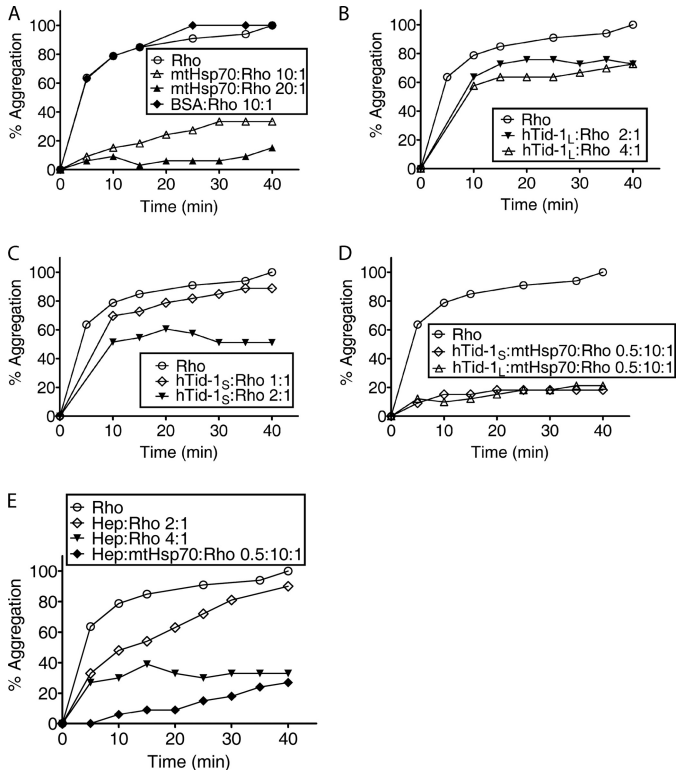

FIGURE 6. **Prevention of aggregation of denatured rhodanese by human mtHsp70 machine components.** Denatured bovine rhodanese in 6 M guanidine hydrochloride was diluted into the reaction mixture and absorbance changes at 320 nm were used to monitor aggregation at different time intervals at 25 °C. Aggregation of denatured rhodanese (0.46 μM) in the presence of, *A,* 4.6 and 9.2 μM human mtHsp70; *B,* 0.92 and 1.84 μM hTid-1_L; *C,* 0.46 and 0.92 μM hTid-1_S; *D,* combination of either 0.23 μM hTid-1_S or hTid-1_L with 4.6 μM human mtHsp70. *E,* aggregation of denatured rhodanese (0.46 μM) in the presence of 0.92 and 1.84 μM Hep alone or 0.23 μM Hep in combination with 4.6 μM human mtHsp70. The aggregation pattern was monitored for 40 min by measuring the changes in turbidity. Percent aggregation values were plotted against time. Under similar conditions, 0.46 μM denatured rhodanese alone was used as an internal control (absence of chaperones) and in the presence of 4.6 μM bovine serum albumin (*BSA*) as a positive control.

(Fig. 6*A*). Compared to DnaK, human mtHsp70 was less robust in providing protection against the aggregation of rhodanese at lower concentrations tested probably due to weaker affinity toward substrates (34).

Similarly, both mitochondrial J-proteins, hTid-1_L and hTid-1_S, showed an ability to prevent aggregation of denatured rhodanese over a range of concentrations. Comparatively, hTid-1_S showed better protection against aggregation at a similar concentration than hTid-1_L (Fig. 6, *B* and *C*). Because, Hep can efficiently stimulate the ATP hydrolysis of mtHsp70, we then tested for its ability to interact with unfolded clients in the presence of different concentrations similar to J-proteins. Surprisingly, as indicated in Fig. 6*E*, greater than 60% protection against aggregation of rhodanese was observed in the presence of 4 times molar excess of Hep. These results provide the first evidence to show that Hep can independently prevent aggregation of client proteins besides its Hsp70 escort activity. To test the aggregation prevention efficiency of mtHsp70 together with J-proteins or Hep as a unit, we performed the experiments using substoichiometric concentrations of hTid-1_S, hTid-1_L, and Hep in combination with mtHsp70. As indicated in Fig. 6, *D* and *E*, a better protection was observed as a unit, indicating their central role in prevention of aggregation of client proteins in the mitochondrial matrix.

Multiple Functional Defects Associated with Human mtHsp70 MDS Mutant—One of the important goals of our reconstitution analysis was to set a platform to dissect specific functional defects associated with the physiologically relevant chaperone mutant phenotypes linked to various mitochondrial disorders in a mammalian system. Recently, several point mutants have been reported in human mtHsp70 that are associated with pathological disorders such as Parkinson disease and myelodysplastic syndrome. To explore the connection between chaperone function and diseased state in the myelodysplastic syndrome, we generated a novel G489E (MDS mutant) point mutant of human mtHsp70 located within the predicted loop (L4 and 5) of the β-sandwich region that is associated with this syndrome (supplemental Fig. S1, *B* and *C*).

The MDS mutant showed a 5-fold elevated basal ATPase activity and 1.6-fold larger K_d value for P5 peptide binding as compared with wild type (Fig. 7*A* and supplemental Table S2). However, the MDS mutant exhibited a severe defect in P5 stimulation even at higher concentrations of substrates in contrast to its peptide affinity (Fig. 7*B* and supplemental Table S2). These results indicated a possible interdomain communication defect associated with this novel mutant, which was not explored previously in other Hsp70s. Similarly, this mutant failed to show stimulation by both J-proteins, hTid-1_S and hTid-1_L (Fig. 7*C, first* and *second panels*). Moreover, human GrpEL1 showed a reduced rate of nucleotide exchange activity with G489E as inhibition was plateaued at 10% hydrolysis in comparison to wild type (compare Fig. 7*D* with Fig. 2*B, upper panel*). Interestingly, however, it retained the ability to be stimulated by Hep as well as showed an enhanced interaction with Hep in both nucleotide states (Fig. 7, *C, third panel*; 4, *B, lanes 11* and *12*). In conclusion, we hypothesize that multiple chaperone-specific biochemical defects associated with the genetic G489E mutant impairs the chaperone cycle, thus leading to MDS.

DISCUSSION

Our major goal in this study was to understand the molecular mechanism of action of various components of the mitochondrial chaperone machine in human mitochondria. To gain insights into the molecular mechanism of mammalian mitochondrial Hsp70 chaperone function, we reconstituted and analyzed the chaperone properties of the human mtHsp70 chaperone machine components utilizing well established *in vitro* biochemical tests. Our analysis reveals four distinct and novel biochemical aspects that are important for understanding the chaperone function of human mtHsp70 and its co-chaperones.

The first aspect reveals a sequence-specific interaction of human mtHsp70 with the peptide substrates derived from mitochondrial targeting sequences of client proteins. Importantly, human mtHsp70 shows very weak affinity toward shorter and less hydrophobic peptide substrates such as P5, whereas it shows higher affinity toward larger peptides that contain more hydrophobic sequences, such as Cox4 when compared with yeast Ssc1. Notably, C-terminal helical lid deletion

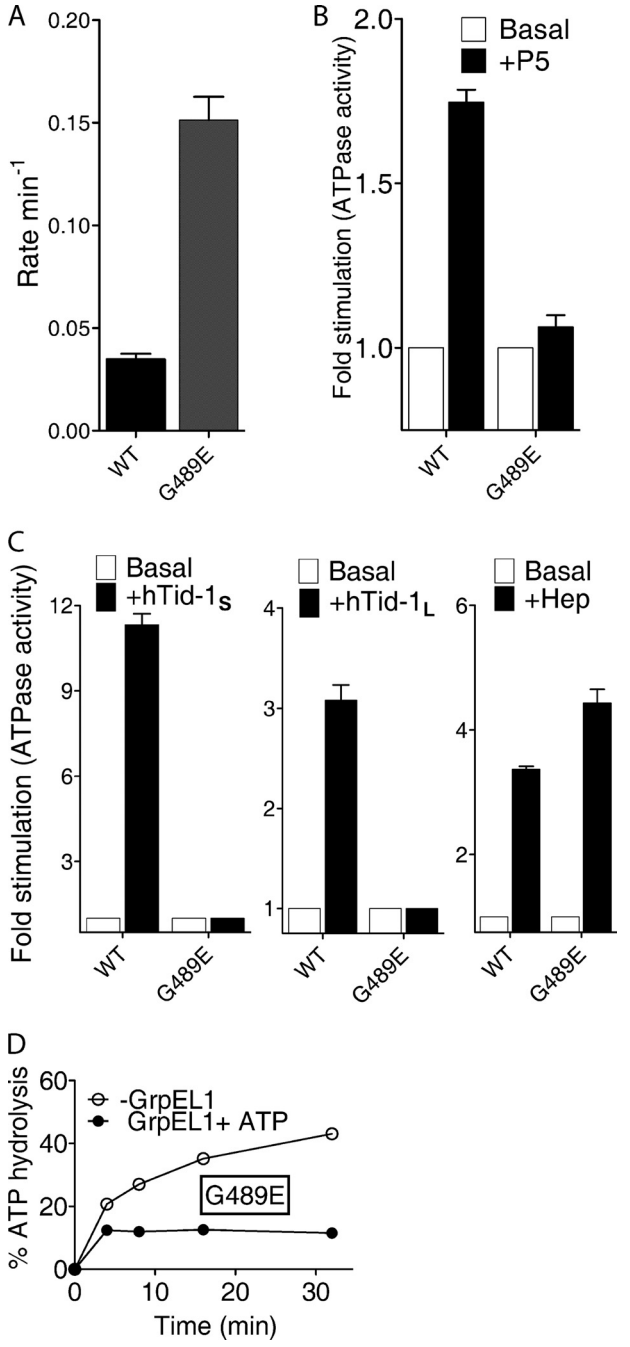

FIGURE 7. **Analysis of biochemical defects associated with MDS mutant (G489E) of human mtHsp70.** 1 μM preformed radiolabeled ATP complexes of wild type (*WT*) and the MDS mutant of human mtHsp70 were incubated with: *A,* buffer alone; *B,* 100 μM P5 substrate; *C,* 1 μM hTid-1_S (*left panel*), 1 μM hTid-1_L (*middle panel*), and 2 μM Hep (*right panel*). Fold-stimulation was calculated by setting the intrinsic ATP hydrolysis rate as 1. *D,* 1 μM preformed MDS mutant-ATP complex was incubated in a buffer alone (*open circles*) and 1 μM GrpEL1 together with 250 μM unlabeled ATP (*closed circles*) at 25 °C. ATP hydrolysis was monitored as a function of time, and percentage of ATP to ADP conversion at various time intervals was plotted. *Error bars* are derived from two independent sets of experiments.

mutants were able to interact with the P5 peptide close to the wild type level, indicating that the SBD of human mtHsp70 possesses a partial open conformation as compared with *E. coli* DnaK and Ssc1. Previously, it has been demonstrated that the lidless variant of DnaK showed 5-fold enhanced k_{off} in ADP-bound state as compared with wild type DnaK, thus promoting it into open conformation (44). However, our data indicates that the lidless variant of human mtHsp70 exhibits ~2.5-fold lower k_{off} for P5 as compared with wild type. Therefore, we speculate that the overall fold and relative orientation of a 6-amino acid shorter helical lid over the SBD of human mtHsp70 significantly differs when compared with other Hsp70s.

The second aspect demonstrates the mechanism of regulation of the chaperone activity of human mtHsp70 by J-protein splice variants: hTid-1_L and hTid-1_S. Our analysis shows that hTid-1_S is more efficient in regulating the ATPase cycle of mtHsp70 due to its robust stimulating activity as compared with hTid-1_L. This raises an intriguing question about the involvement of 33 amino acids from the C-terminal end of hTid-1_L in negatively regulating its ability to stimulate the ATPase activity of mtHsp70. On the other hand, it is possible that an insertion of 6 new amino acids at the C terminus of hTid-1_S leads to gain of function, thus stimulating more efficiently. Also, both J-protein isoforms displayed differential abilities in preventing aggregation of denatured rhodanese. The functional differences between these two J-protein variants may be the primary reason for opposite phenotypes seen at the cellular level as reported (13).

Third, our analysis uncovers a detailed novel mechanism in which the Hep protein modulates the chaperone function of human mtHsp70. Our observation provides the first direct evidence showing that the stability of Hep interaction is also dependent on the C-terminal region of human mtHsp70. Based on our GST pulldown analysis, we hypothesize that C-terminal domain α-helices C, D, and E are directly involved in negatively regulating the Hep interaction in wild type protein. This is supported by two important observations. First, the truncation of C to E α-helices enhances the interaction of Hep in the case of the M600 deletion mutant. Second, a restoration of the wild type level of interaction in M584 and M555 deletion mutants suggests that amino acids from 584 to 600 might be critical for Hep binding at the C terminus of human mtHsp70. Similarly, an enhanced interaction with Hep was also observed in arch or SBD human mtHsp70 cleft mutants due to retention of C-terminal contact sites, comprised of amino acids 584 to 600 in these mutants. However, the negative regulation by C-terminal C, D, and E α-helices may largely be ineffective in mutant proteins due to alteration in the positioning of these helices relative to the β-sandwich domain.

On the other hand, we do not rule out the possibility that deletion of α-helices (C, D, and E) may overall influence the relative orientation of the SBD and ATPase domain of human mtHsp70 by altering the position of the interdomain linker region, thus promoting a favorable conformation for better Hep binding in M600 and arch/cleft point mutants. Recent experimental evidences are in favor of this hypothesis wherein the interdomain linker region has been shown to play a critical role for binding Zim17 to the ATPase domain of mtHsp70 in yeast (45). Such rearrangements in the domain interface in mutants may promote a higher propensity to generate self-aggregation prone conformers in non-nucleotide or the ATP-bound state thereby enhancing their binding with Hep protein. Also, the functional significance of C-terminal D and E α-helices is well established in bacterial DnaK (46). Our result emphasize the

importance of C, D, and E α-helices in regulating the interaction of Hep with mtHsp70 in human mitochondria.

The nature of Hep interaction at the C terminus of wild type human mtHsp70 is unique and distinct from substrates or J-proteins. Two biochemical evidences presented here support our hypothesis. 1) The preformed Hep-human mtHsp70 complex in the presence or absence of nucleotides is not destabilized by excess levels of P5 peptide, indicating its different nature of interaction at the C terminus. 2) All deletion and point mutants of human mtHsp70 significantly retained their ability to stimulate ATPase activity as compared with J-proteins. However, the increased Hep interaction with the human mtHsp70 mutants did not show significant enhancement in stimulation of ATPase activity. We speculate that the physical interaction through the C terminus of human mtHsp70 is dispensable for modulating the conformational changes necessary for activation of the ATPase domain to hydrolyze the ATP.

Interestingly, despite the absence of J-domain, Hep showed a unique ability to stimulate ATPase activity of human mtHsp70. However, we conclusively rule out the possibility of Hep functioning as a nucleotide exchange factor, as speculated earlier based on the stimulatory activity of Hep observed in single turnover experiments (26). Furthermore, our biochemical data demonstrate that the mechanism of Hep action closely resembles the type-I J-protein function. To further confirm the specificity of stimulation, we have identified critical amino acid residues of the N terminus of human mtHsp70 that are essential for inducing the conformational changes during ATP hydrolysis. Surprisingly, these residues were found canonical with those that are essential for J-protein stimulation by DnaJ and hTid-1_S for *E. coli* DnaK and human mtHsp70, respectively. Besides, binding of hTid-1_S can be competed out by Hep or *vice versa*, indicating that the interaction sites of these two proteins at the ATPase domain of human mtHsp70 are mutually exclusive. The overlap in binding sites between Hep and hTid-1_S indicates that they may induce similar conformational changes in the ATPase domain that results in acceleration of ATP hydrolysis. However, the rate at which they couple conformational changes to the ATP hydrolysis in the chaperone cycle might differ due to differences in ATPase stimulation activities between them.

Besides interacting with Hsp70 partner proteins, our *in vitro* biochemical experiments reveal that Hep also possesses human mtHsp70-independent functions. For example, Hep exhibits *bona fide* chaperone activity, because it can bind unfolded substrates such as rhodanese, preventing its aggregation even in the absence of the Hsp70 partner protein similar to type-I J-proteins. Based on these chaperone-specific functions performed by Hep, we propose that Hep represents a member of a new class of co-chaperone for Hsp70 evolved for higher eukaryotic mitochondria biogenesis. However, the specific mitochondrial client protein other than Hsp70 requiring the assistance of Hep to prevent aggregation in response to various physiological stress stimuli is yet to be elucidated.

The fourth aspect focuses on understanding the chaperone-specific functional defects associated with MDS mutant. The mutant shows significant defects in interacting with J-protein co-chaperones (hTid-1_S and hTid-1_L) as well as the reduced rate of nucleotide exchange ability by GrpEL1. The basal ATPase activity is significantly elevated and together with the loss of stimulation by client peptides indicates an interdomain communication defect associated with this novel loop mutant. Therefore, we hypothesize that the loss of mtHsp70 activity in the MDS mutant impedes the import of many precursor proteins and their subsequent folding in the matrix leading to mitochondrial dysfunction. Our results establish that the loss of chaperone function may be the leading cause of myelodysplastic syndrome. To evaluate the importance of this residue in other Hsp70s, we made a similar mutation at the corresponding position in yeast Ssc1, which resulted in a lethal phenotype signifying the importance of this residue in the proper functioning of mtHsp70.[3] Our biochemical insights will provide further understanding of this syndrome at the physiological level.

In summary, our results establish and highlight several unique and distinct biochemical features of the human mitochondrial chaperone machine (mtHsp70/J-protein/GrpE/Hep) that are critically required for protein quality control in the mitochondrial matrix. Additionally, it confirms the need for multiple co-chaperones for proper mitochondria biogenesis required for fulfilling cellular demands in the mammalian system. Besides, our investigation also provides key insights to connect the involvement of chaperone function in a diseased state such as myelodysplastic syndrome. Together, our results provide a better platform for the future investigation on mtHsp70-based therapeutic design in treating various mitochondrial disorders.

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