Saccharomyces cerevisiae Hsp104 Enhances the Chaperone Capacity of Human Cells and Inhibits Heat Stress-Induced Proapoptotic Signaling†

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ABSTRACT: Hsp104, the most potent thermotolerance factor in *Saccharomyces cerevisiae*, is an unusual molecular chaperone that is associated with the dispersal of aggregated, non-native proteins in vivo and in vitro. The close cooperation between Hsp100 oligomeric disaggregases and specific Hsp70 chaperone/cochaperone systems to refold and reactivate heat-damaged proteins has been dubbed a "bichaperone network". Interestingly, animal genomes do not encode a Hsp104 ortholog. To investigate the biochemical and biological consequences of introducing into human cells a stress tolerance factor that has protein refolding capabilities distinct from those already present, Hsp104 was expressed as a transgene in a human leukemic T-cell line (PEER). Hsp104 inhibited heat-shock-induced loss of viability in PEER cells, and this action correlated with reduced procaspase-3 cleavage but not with reduced c-Jun N-terminal kinase phosphorylation. Hsp104 cooperated with endogenous human Hsp70 and Hsc70 molecular chaperones and their J-domain-containing cochaperones Hdj1 and Hdj2 to produce a functional hybrid bichaperone network capable of refolding aggregated luciferase. We also established that Hsp104 shuttles across the nuclear envelope and enhances the chaperoning capacity of both the cytoplasm and nucleoplasm of intact cells. Our results establish the fundamental properties of protein disaggregase function in human cells with implications for the use of Hsp104 or related proteins as therapeutic agents in diseases associated with protein aggregation.

Exposure of cells or organisms to a mild heat shock leads to a transient state of increased heat resistance (*1*). This thermotolerant state is attributed to the increased capacity of cells with elevated levels of heat-shock proteins to prevent irreversible protein misfolding (reviewed in ref *2*). In animal cells, elevated heat-shock-protein expression prevents stress-induced apoptosis (*3*), and disruption of the HSF1 gene, which encodes the transcription factor responsible for stress-induced expression of the heat-shock-protein genes, results in an inability to develop thermotolerance and an increased sensitivity to stress-induced apoptosis (*4*). While the mechanisms of heat-shock-protein-mediated regulation of apoptosis remain unclear, considerable evidence implicates the blockade of specific biochemical events in the apoptotic signaling pathway by Hsp70 (*5*) and small heat-shock protein, Hsp27 (*6*).

In yeast, neither reduction of Hsp70 expression (*7*) nor elimination of the small heat-shock protein Hsp26 (*8*) have a substantial effect on survival of yeast exposed to severe heat stress. Instead, thermotolerance in yeast is highly dependent on the expression of a different molecular chaperone, Hsp104 (*9*). Hsp104 is required for the recovery of heat-inactivated bacterial luciferase solubility and enzyme activity in intact yeast cells, as well as the dispersal of heat-induced aggregates (*10*). Reconstitution of Hsp104-dependent refolding in vitro established that, in contrast to other chaperone systems, Hsp104 specializes in reactivating proteins trapped in aggregates and that cannot be efficiently reactivated by other chaperone systems (*11*). Nonetheless, Hsp104 and its relatives, yeast mitochondrial Hsp78 and bacterial ClpB, do not function productively in protein reactivation alone, but each requires specific Hsp70s and Hsp70 cochaperones to disaggregate and refold target proteins (*11*−*13*) in a functional interaction called a "bichaperone network" (*13*).

Genes encoding orthologs of Hsp104 are found in the genomes of fungi, plants, and bacteria and, like Hsp104 in yeast, Hsp101 in *Arabidopsis* (*14*, *15*) and maize (*16*) and ClpB in *Escherichia coli* (*17*, *18*) are all important thermotolerance determinants. Intriguingly, Hsp104 orthologs are absent from animal genomes and there is no evidence that they possess a bichaperone network analogous to the Hsp104/Hsp70 network in yeast that is capable of reversing protein aggregation. Thus, the expression of Hsp104 in animal cells represents a novel opportunity to analyze the properties of a distinct chaperone capability in cells that lack the equivalent machinery and to ascertain its impact on survival.

As a host for Hsp104 expression, we chose a human leukemic T-cell line (PEER) that has been used extensively

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as a model of heat-shock-induced apoptosis (*3*, *19*−*21*). When expressed from a tetracycline-inducible promoter, wild-type Hsp104, but not a mutant form of the protein, enhances the viability of heat-shocked PEER cells and this effect correlates with diminished cleavage of procaspase-3. We find that Hsp104 forms a hybrid bichaperone network with human Hsp70 chaperones and DnaJ-like cochaperones. Furthermore, we establish that Hsp104 shuttles across the nuclear envelope in human cells and is active in protein refolding in both the cytoplasm and nucleoplasm.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines. Hsp104 open-reading frame was excised from the yeast expression vector pLA28SX (provided by S. Lindquist, Whitehead Institute) as a *Bam*HI−*Sac*I fragment and subcloned into pCDNA3 (Invitrogen). For inducible expression, the Hsp104-coding sequence was excised from pCDNA3Hsp104 as a *Sac*I fragment, blunted with T4 DNA polymerase and inserted into the tetracycline-regulated dicistronic expression plasmid, pTR5-DC/GFP*tk/hygro (*21*). The second cistron of the resulting plasmid (pTR5-DC/Hsp104-GFP*tk/hygro) encodes GFP.[1] GFP-positive cells were selected by micromanipulation and expanded (*22*). Expression of Hsp104 was confirmed by Western blot analysis. An inducible expression vector encoding an ATPase-deficient derivative of Hsp104 (Hsp104K218T; ref *23*) was constructed using identical methods. Cell lines expressing wild-type and mutant Hsp104 were designated PrtTAHsp104 and PrtTAHsp104K218T, respectively. The plasmids, pRSVcyt-Luc and pRSVnuc-Luc, encoding firefly luciferase (FFL) lacking a C-terminal peroxisomal targeting signal for cytoplasmic localization or containing a SV40 large T antigen nuclear localization signal for nucleoplasmic accumulation (*24*) were provided by H. Kampinga and were transfected into PrtTAHsp104 and PrtTAHsp104K218T by electroporation for in vivo refolding experiments. HEK293 cells were transfected with pCDNA3Hsp104 for the subcellular localization experiment.

Cell Viability Assays. Cultures in mid-log phase were split and DOX (1 μg/mL) was added to one-half. After 24 h, both induced and uninduced cells were washed with PBS and resuspended in fresh media containing 20 mM HEPES-KOH at pH 7.2. Cells were heat-shocked by immersion in a 43 °C water bath for the indicated times; control cells remained at 37 °C. Following heat shock, cells were diluted with fresh medium and returned to a 37 °C CO_2 incubator for 24 h. Cell viability was measured by the reduction of MTT according to the instructions of the supplier (ATCC). Viability was calculated as the ratio of color development for heat-shocked cells relative to that of nonheat-shocked cells in each cell line examined.

Cell Extracts. As required, Hsp104 expression was induced with 1 μg/mL DOX for 48 h. Cells were harvested, washed in 1× refolding buffer (RFB; 25 mM HEPES-KOH at pH 7.6, 150 mM potassium acetate, 10 mM magnesium acetate, and 10 mM DTT), flash-frozen in liquid nitrogen, and stored at −80 °C until use. Thawed cells were resuspended in two volumes of RFB supplemented with 1 mM AEBSF and 2 μg/mL each of aprotinin, leupeptin, and pepstatin A. Crude lysates were prepared by sonication and clarified by centrifugation at 100000*g* for 30 min at 4 °C. For ion exchange chromatography, PEER cells were suspended in two volumes of extraction buffer consisting of 20 mM Tris-HCl at pH 8.0, 10 mM DTT, and protease inhibitors. Cells were disrupted with a motorized Potter−Elvehjem homogenizer. A total of 20 mg of clarified extract was applied to a 1-mL Resource Q column (AP Biotech), washed with 5 column volumes of extraction buffer, and eluted in the same buffer with a linear gradient of 0−500 mM NaCl over 20 column volumes collecting 1-mL fractions. Fractions were dialyzed against 1× RFB before being used in the refolding assays.

Luciferase Refolding Assays (in Vitro). Aggregated FFL was prepared as previously described (*11*). To avoid batch-to-batch variation in substrate preparation, aliquots of aggregated FFL were flash-frozen in liquid N_2, stored at −80 °C, and thawed on ice immediately prior to use. Each refolding reaction (50 μL final volume) contained 5 μL of aggregated FFL (20 nM final concentration), 31 μL of dialyzed column fraction or cell extract, 5 mM ATP, 25 mM phosphocreatine, 100 mU of creatine phosphokinase, and 0.1 mg/mL BSA, supplemented with the indicated purified chaperones at a final concentration of 1 μM with respect to the monomers. After incubation for 90 min at 25 °C, 1 μL of each refolding reaction was diluted into 50 μL of Luciferase Assay Reagent (Promega) and light emission was measured for 5 s with a luminometer (Berthold LB9507).

Luciferase Refolding Assays (in Vivo). Following transformation with pRSVcyt-Luc and pRSVnuc-Luc, cells were cultured for 24 h in medium containing 200 μg/mL hygromycin and 150 μg/mL G418 and split. One-half was induced with 3 μg/mL DOX for 14 h. Harvested cells were resuspended in medium containing 30 μg/mL cycloheximide and 20 mM MOPS at pH 7.0. Submerging the cells in a 44 °C water bath for precisely 6 min inactivated FFL. The cells were then maintained at room temperature for an additional 6 h. FFL activity was determined as previously described (*25*).

Protein Purification. Human Hdj1/Hsp40 (SPP400) and Hsc70/Hsp73 (SPP751) were purchased from Stressgen. Recombinant Hsp104, human Hsp70, and His-tagged Hdj2 were purified following expression in *E. coli* using standard chromatographic procedures.

Immunofluorescence. HEK293 cells transfected with pCDNA3Hsp104 were seeded onto sterile coverslips and permitted to grow for 2 days. Cells were rinsed with PBS and fixed with 4% formaldehyde for 20 min followed by treatment with 50 mM NH_4Cl for 5 min. Cells were permeabilized with 0.2% (v/v) TX-100 in PBS. After blocking, cells were probed with polyclonal rabbit anti-Hsp104 antibody diluted 1:10 000 in the blocking solution followed by Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories) for 1 h and finally treated with 1 μg/mL DAPI for 5 min. To examine CRM1-dependent trafficking, cells were treated with 10 ng/mL leptomycin B (Sigma) for 4 h in the presence of 20 μg/mL cycloheximide. To examine the effect of heat shock on protein localization, transfected cells were grown on sterile chamber slides (Lab-Tek).

[1] Abbreviations: GFP, green fluorescent protein; DTT, dithiothreitol; AEBSF, 4-(2-aminoethyl)benzene sulfonylfluoride; DAPI, 4′,6-diamidino-2-phenylindole; LMB, leptomycin B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; FFL, firefly luciferase; DOX, doxycycline, JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated kinase.

Growth medium was replaced with a medium buffered with 20 mM HEPES-KOH at pH 7.0, and the slides were heated to 43 °C for 30 min and returned to 37 °C in fresh growth medium for 4 h. Cells were immunostained for Hsp104 as described above. Hsc/p70 was detected with a 1:500 dilution of a mouse monoclonal antibody (Sigma, Clone BRM-22) in combination with a Cy5-conjugated donkey anti-mouse IgG secondary (Jackson Laboratories). Cells were imaged with a standard fluorescence microscope (Zeiss Axioskop) equipped with a Spot Junior CCD camera (Diagnostics Instruments).

Antibodies. The following antibodies were used in these experiments: rabbit polyclonal anti-Hsp104, rabbit polyclonal anti-Hdj1 (Hsp40, Stressgen, SPA400); mouse monoclonal anti-Hdj2 (Neomarkers, Clone KA2A5.6); rat monoclonal anti-Hsc70 (Hsp73, Stressgen, SPA815); mouse monoclonal anti-Hsp70 (Stressgen, SPA810); rabbit polyclonal anti-Hsp70 (Hsp72, Stressgen, SPA812); mouse monoclonal anti-Hsp70 (Sigma, BRM-22); rabbit polyclonal anti-caspase 3 (a gift from D. W. Nicholson, Merk Frosst, Pointe Claire, Quebec, Canada), rabbit polyclonal anti-activated c-Jun N-terminal kinase (JNK) (pTPpY, Promega); and mouse monoclonal anti-actin (ICN, C4). Immunocomplexes were detected by enhanced chemiluminescence.

RESULTS

Hsp104 Enhances Thermoresistance of Human Cells. To determine if Hsp104 confers thermoprotection when expressed in human cells, we compared the rate of viability loss in tetracycline-induced and uninduced PEER cells subjected to a heat shock of 43 °C. We analyzed in parallel the thermoresistance of cell lines expressing Hsp104K218T, an ATPase-dead derivative of Hsp104 (*23*) or human Hsp70. As previously observed (*21*), tetracycline-induced expression of Hsp70 enhanced the thermoresistance of PEER cells (Figure 1). Wild-type Hsp104 expression but not the expression of Hsp104K218T also enhanced thermoresistance of heat-shocked cells compared to uninduced controls, indicating that the influence of Hsp104 depended on the intrinsic ATPase activity of the chaperone and not simply on the presence of the transgene or the protein itself. Western blot analysis confirmed that Hsp104 and Hsp104K218T were expressed at similar levels in tetracycline-induced cells (Figure 2). In the absence of tetracycline-induced Hsp70 expression, heat-shock-induced accumulation of Hsp70 increased as a function of time of recovery at 37 °C and the expression of either Hsp104 or mutant Hsp104 had little impact on endogenous Hsp70 expression.

In heat-shocked PEER cells, the appearance of condensed chromatin, caspase 3 and caspase 9 activation, DEVDase activity, poly(ADP-ribose) polymerase cleavage, JNK activation, annexin V binding, and cytochrome *c* release have all been reported previously (*3*, *20*, *21*). Each of these apoptosis-associated responses is inhibited by the overexpression of Hsp70. To determine if Hsp104 might similarly inhibit apoptotic signaling, we examined two indicators: JNK phosphorylation and procaspase-3 cleavage. As previously observed, Hsp70 strongly inhibited the transient activation of JNK that is evident immediately after heat shock (Figure 2). In addition, Hsp70 expression inhibited procaspase-3 cleavage detected during recovery in control cells by the

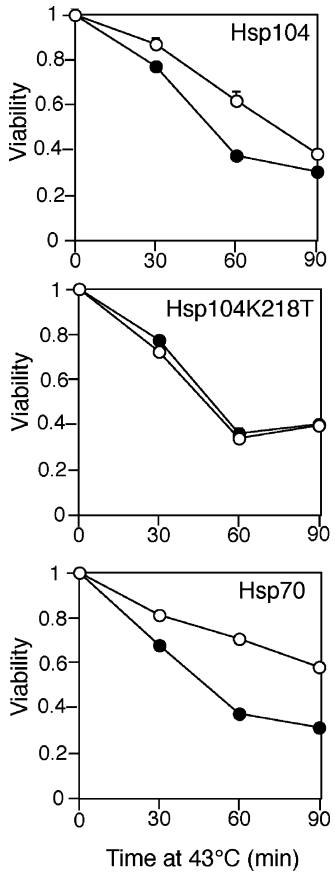

FIGURE 1: Hsp104 expression enhances viability of PEER cells following heat shock. PEER cells expressing yeast Hsp104, an ATPase deficient mutant Hsp104K218T, and human Hsp70 were untreated (●) or induced for 24 h with 1 μg/mL DOX (○). Cells were heat-shocked at 43 °C for the indicated times and then returned to 37 °C. After 24 h, viability was determined by MTT assay. Plotted points represent the means of three independent experiments (error bars = standard error of the mean).

appearance of a 17 kDa cleavage product. Hsp104 also inhibited procaspase-3 cleavage but to a lesser extent than Hsp70, while Hsp104K218T had no discernible effect. Importantly, in contrast to the effect of Hsp70, Hsp104 had no influence on the activation of JNK.

Subcellular Distribution of Hsp104 in Human Cells. Immunoelectron microscopy indicates that at least some Hsp104 is present in the nuclei of yeast (*26*). Because the mass of assembled Hsp104 (~600 kDa) is too large to translocate through the nuclear pore complex by passive diffusion, entry into the yeast nucleus is likely mediated by nuclear transport factors. To determine if Hsp104 can enter the nucleus in mammalian cells, we examined its distribution using indirect immunofluorescence. PEER cells are round, nonadherent cells in which the cytoplasm forms only a thin layer around the nucleus. We therefore used an adherent cell line, HEK293, transiently transfected with pCDNA3Hsp104 for these experiments. In transfected cells, Hsp104 appeared to be distributed throughout the cell including the nucleoplasm as indicated by overlapping staining with DAPI (Figure 3A).

In mammalian cells, it is possible that some Hsp104 is simply entrapped in the nucleus during reassembly of the nuclear envelope following mitosis. We therefore examined the effect of leptomycin B (LMB), a covalent modifier of the nuclear export factor Crm1 (*27*), on Hsp104 localization.

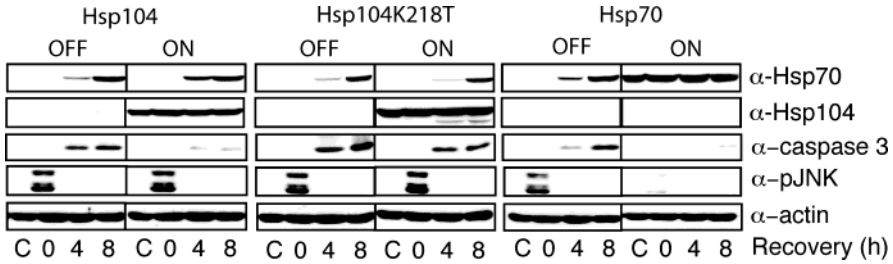

FIGURE 2: Hsp104 expression inhibits caspase 3 activation. PEER cell lines, untreated or induced with DOX as indicated, were heat-shocked for 1 h at 43 °C. Cells were collected before heat shock (C), immediately after heat shock (0), and after 4 and 8 h of recovery at 37 °C. Equal amounts of cell lysate were subjected to SDS−PAGE, blotted, and probed with the indicated antibodies. In anti-caspase 3 blots, only the 17 kDa cleavage product is shown. The anti-actin blot indicates equal loading of the lanes.

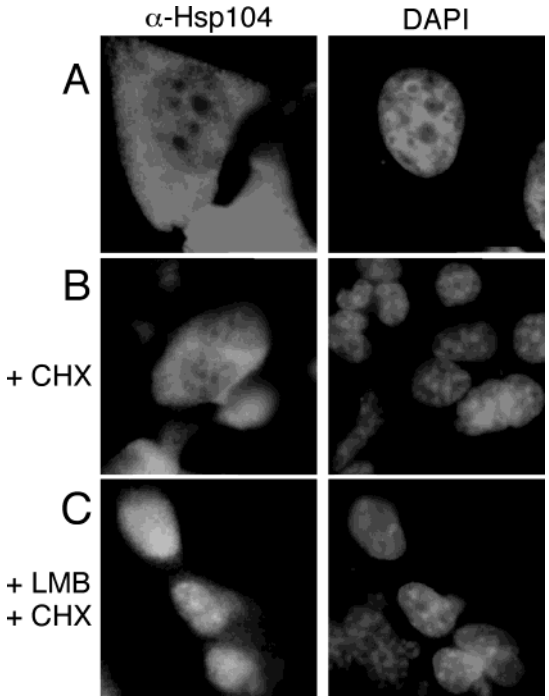

FIGURE 3: Hsp104 shuttles across the nuclear envelope in human cells. HEK293 cells were cultured on coverslips and transfected with pCDNA3Hsp104. After 24 h, post-transfection cells were fixed and permeablilized. Hsp104 was detected with an anti-Hsp104 polyclonal serum (left column), and nuclear DNA was stained with DAPI (right column). Cells were untreated (A) or, to prevent synthesis of Hsp104 in the cytosoplasm, treated with 20 μg/mL cycloheximide alone (CHX; B) or together with 20 ng/mL LMB (C) and incubated at 37 °C for 4 h prior to fixation.

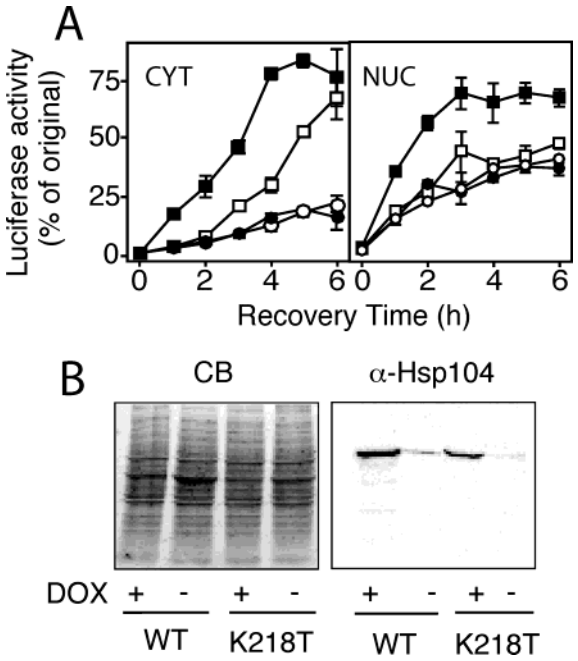

FIGURE 4: Hsp104 enhances in vivo refolding of thermally inactivated FFL in the nucleoplasm and cytoplasm of intact cells. (A) PrtTAHsp104 (□ and ■) and PrtTAHsp104K218T (○ and ●) cell lines were transfected by electroporation with plasmids that direct the expression of cytoplasmic- (CYT) or nucleoplasmic-localized (NUC) FFL and were either left untreated (□ and ○) or induced with DOX for 24 h (■ and ●). FFL was inactivated by heating at 44 °C for 6 min. Recovered FFL activity was normalized to the level of activity measured in an equivalent number of cells immediately before heat inactivation. Error bars = standard deviation of three FFL determinations at each time point. (B) Cell extracts were analyzed by SDS−PAGE and stained with Coomassie blue (CB) to indicate equal loading of protein. A duplicate gel was blotted with anti-Hsp104 antibody. A representative result of three independent trials is shown.

To prevent the synthesis of Hsp104 in the cytoplasm during the LMB treatment, cells were also treated with cycloheximide. The majority of cells receiving only the cycloheximide treatment showed the same distribution of Hsp104 as cells receiving no treatment (Figure 3B). In contrast, most cells that were treated with LMB and cycloheximide showed distinctly more intense nuclear staining for Hsp104 and depletion of cytoplasmic Hsp104, a pattern that is characteristic of many shuttling proteins (Figure 3C).

In mammalian cells, heat shock induces the transient redistribution of Hsp70 to the nucleus (*28*). Indeed, after mild heat shock and recovery, immunostaining for Hsp70 revealed that these proteins accumulated in the nucleus of a substantial number of the cells but the pattern of Hsp104 distribution was unchanged (data not shown).

Hsp104 Enhances Protein Reactivation in the Cytosol and Nucleus. The ability of Hsp104 to promote survival of heat-shocked PEER cells correlated with its inhibition of pro-caspase-3 cleavage. To determine if Hsp104 expression also contributes in a more general way to the protein refolding capacity in intact PEER cells, we examined the reactivation of heat-denatured FFL mistargeted to either the cytoplasm or nucleoplasm as a probe of chaperone function in vivo. Both the cytoplasmic and nucleoplasmic FFL activities were more rapidly restored in Hsp104-expressing cells than in either uninduced cells or cells expressing Hsp104K218T (Figure 4A). Unexpectedly, reactivation of cytosolic FFL in uninduced PrtTAHsp104 cells was observed in repetitions of the experiment albeit delayed by about 2 h relative to the induced cells. It is plausible that the low but detectable level of Hsp104 expressed in uninduced cells (Figure 4B) con-

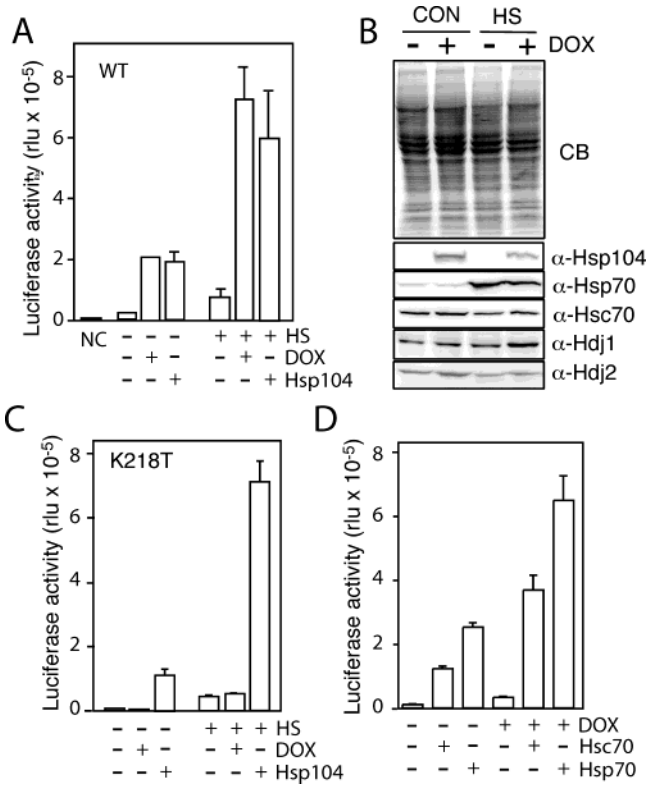

FIGURE 5: Hsp104-mediated refolding is stimulated by heat-shock induction of endogenous chaperones. Cell-free protein extracts were prepared from PrtTAHsp104 cells. (A) Reactions (25 μL) containing 74 μg of total soluble protein (100000*g* supernatant) from each lysate was used to refold non-native aggregated FFL. The indicated reactions were supplemented with 0.2 μM recombinant Hsp104. FFL activity was determined after 2 h of refolding at 25 °C. (B) Each lysate (35 μg) was loaded on duplicate SDS−PAGE gels. CB staining indicates equal loading. Duplicate gels were blotted, and the membranes were probed with the indicated antibodies. (C) Cell-free extracts were prepared from PrtTAHsp104K218T cells and used to refold FFL as described for A. (D) Extracts were prepared from PrtTAHsp104 cells and supplemented as indicated with 2 μM Hsc70 or Hsp70 and used to refold aggregated FFL. All refolding assays were conducted three times.

tributed to the slow reactivation of cytoplasmic FFL that we observed.

Hsp104 and Human Chaperones Form a Hybrid Bichaperone Network. Having established that Hsp104 provides thermoresistance and enhanced chaperone activity in vivo, we examined whether Hsp104 functioned in conjunction with the endogenous chaperones of human cells to form a hybrid "bichaperone network" or simply acted as an orphan molecular chaperone. Total soluble protein extracts were prepared from uninduced or tetracycline-induced PrtTAHsp104 cells maintained at 37 °C or heat-shocked at 43 °C and permitted to recover for 8 h. The chaperone capacity of these lysates was measured by the refolding of chemically denatured, aggregated FFL. In the absence of Hsp104 induction, the yield of refolded FFL was low but was nonetheless enhanced in extracts from heat-shocked cells (Figure 5A). Even though the FFL used as a substrate in these reactions was preaggregated, it has been previously shown that the size of aggregates prepared in this manner are heterogeneous (*11*). Very low molecular weight aggregates within such a mixture can be remodeled by Hsp70/40 chaperone systems without the cooperation of an Hsp100 disaggregase (*11*, *29*). Thus, the increase in the yield of refolded luciferase was enhanced by the heat-induced expression of Hsp70 detected by Western blot analysis of the lysates (Figure 5B).

The yield of refolded FFL was dramatically higher in extracts derived from induced cells, consistent with the observation that refolding of FFL trapped in higher molecular weight aggregates is largely dependent on Hsp104 (*11*). The yield of FFL refolded by heat-shocked cell lysate was 3-fold higher compared to that by unheated control cell lysate, indicating that Hsp104 itself is activated by heat shock or that other heat-inducible host cell factors enhance the function of Hsp104. To rule out the possibility that the apparent increase in Hsp104-dependent refolding was due to an increase in the activity of Hsp104 itself, recombinant Hsp104 (0.2 μM final concentration) was added to extracts of uninduced cells. Indeed, the enhancement of exogenous Hsp104 function in heat-shocked extracts paralleled the effect of heat shock on transgenically expressed Hsp104 suggesting that heat shock alters the ability of the host cell to support Hsp104-mediated refolding.

Hsp104 participates in a bichaperone network with cytosolic Hsp70s encoded by the *SSA1*−*4* genes in yeast and either of two Hsp70 cochaperones, Ydj1 (*11*) or Sis1 (*12*). We therefore probed the cell extracts with antibodies that specifically detect the human orthologs of the relevant yeast chaperones to determine which, if any, might be involved in enhancing Hsp104 function (Figure 5B). This analysis indicated that the most significant change in endogenous chaperone levels was in heat-shock-inducible Hsp70.

Furthermore, to establish that the reduced chaperone activity in intact cells expressing Hsp104K218T was attributable to the presence of inactive Hsp104 rather than a different cause, refolding reactions were carried out using extracts prepared from uninduced and induced PrtTAHsp104K218T cells. As anticipated, the induction of Hsp104K218T did not influence the refolding capacity of these lysates, (Figure 5C) but supplementation with recombinant Hsp104 restored refolding to levels obtained using lysate from induced PrtTAHsp104 cells. Refolding in uninduced extracts of PrtTAHsp104 cells, while very limited, was substantially higher than in the equivalent extract from PrtTAHsp104K218T cells. This observation supports the idea that leaky expression of active Hsp104 could account for the delayed refolding observed in intact uninduced PrtTAHsp104 cells. Unfortunately, refolding reactions in vitro cannot be sustained for the several hours required to mimic more closely the in vivo refolding experiment.

To determine if the enhancement of Hsp104 function in cell extracts of heat-shocked cells represented simply a general increase in total Hsp70 (a combination of constitutively expressed Hsc70 and heat-inducible Hsp70) or a specific functional interaction between Hsp104 and heat-inducible Hsp70, PrtTAHsp104 lysates were prepared from nonheat-shocked cells with or without induction of Hsp104 expression. These extracts were supplemented with either purified Hsc70 or Hsp70 in excess (2 μM) and used in refolding reactions. Either in the presence or absence of Hsp104 induction, both proteins enhanced luciferase refolding in cell-free extracts, although Hsc70 was less effective than Hsp70 (Figure 5D).

We recapitulated Hsp104-dependent refolding with purified Hsp104 and human chaperones. All reactions contained

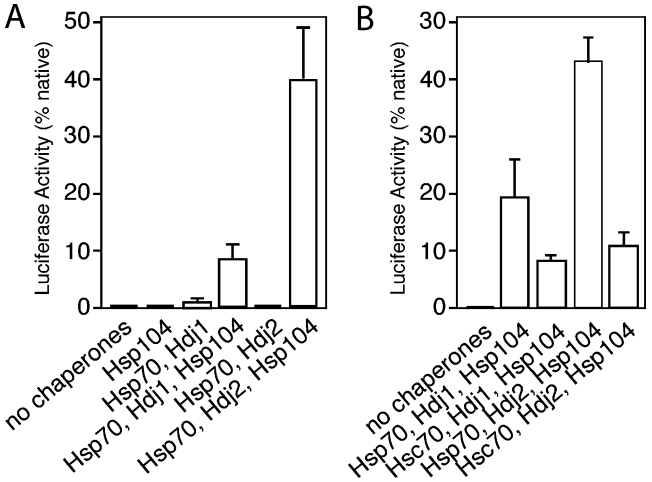

FIGURE 6: Yeast Hsp104 functions with purified human molecular chaperones in a hybrid bichaperone network. Refolding of aggregated FFL was measured in reactions containing purified human molecular chaperones with or without purified yeast Hsp104. FFL activity was determined after 90 min of refolding and expressed as a percentage of input of native FFL activity. (A) Comparison of refolding in reactions containing human Hsp70 with either human Hdj1 or Hdj2. (B) Comparison of refolding by purified Hsp70 and Hsc70.

1 μM of each protein (monomers). We first examined refolding by recombinant human Hsp70 with either of two alternative J-domain-containing cochaperones, Hdj2 or Hdj1 (Figure 6A). Hsp70, Hdj1, or Hdj2 alone or in combination with Hsp104 resulted in substantial refolding above the spontaneous level measured in reactions lacking chaperones (data not shown). As anticipated for refolding of aggregated substrate, Hsp70 with either cochaperone refolded only limited amounts of FFL in the absence of Hsp104. Hsp104 by itself was also ineffective in refolding, but together with human Hsp70 and either cochaperone, we observed a substantial yield of refolded FFL suggesting that these proteins constitute a hybrid bichaperone network. Hsp104-dependent refolding with Hsp70 and Hdj1 was less effective than that observed with Hsp70 and Hdj2, while the limited but reproducible refolding by Hsp70 and Hdj1 in the absence of Hsp104 was slightly better. Hsp70 could be replaced by Hsc70 in Hsp104-dependent refolding reactions (Figure 6B).

Do Mammalian Cells Possess an Endogenous Bichaperone Network? The two elements of a bichaperone network are a disaggregase that acts on non-native aggregated proteins and an Hsp70/Hsp40 chaperone/cochaperone system that assists in refolding proteins released from aggregates. Especially because the supplementation of lysates with Hsp70 resulted in substantial refolding of aggregated luciferase (Figure 5D), we wanted to determine whether Hsp104 provides a unique protein refolding capability when expressed in human cells or whether these cells might contain an endogenous factor that could substitute for Hsp104 activity in the refolding of aggregated proteins. We prepared extracts from uninduced or tetracycline-induced PrtTAHsp104 cells. The extracts were fractionated by anion exchange chromatography, and each fraction was tested for its ability to refold previously aggregated FFL alone or with the addition of human Hsp70 and Hdj2. The extent of refolding was normalized to reactions containing just purified chaperones Hsp104, Hdj2 and Hsp70. Robust refolding was detected only in the fractions from cells expressing Hsp104 and only when

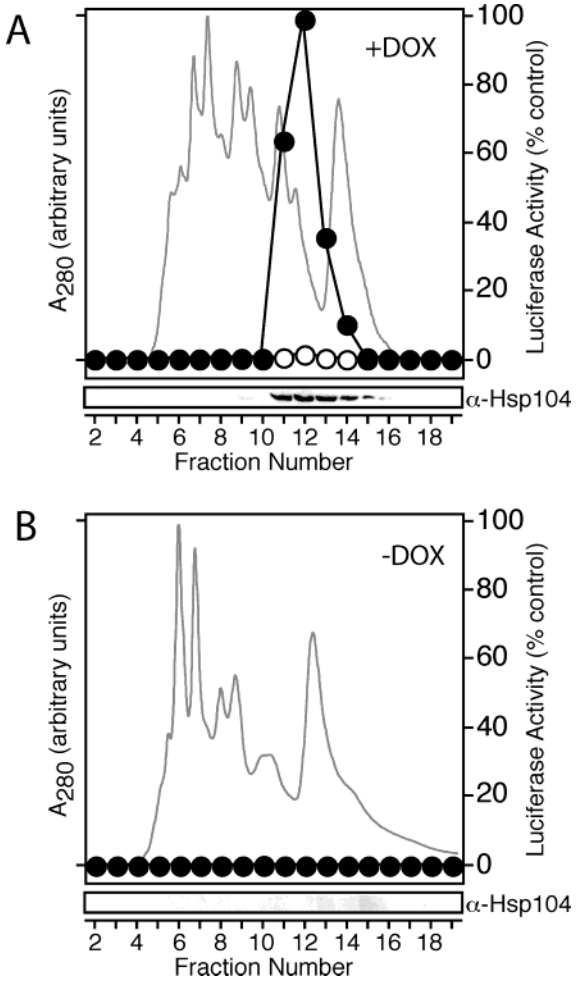

FIGURE 7: Hsp104 is the only potent disaggregase present in cell-free lysates. Uninduced and induced PrtTAHsp104 cells were used to prepare cell-free lysates. A total of 20 mg of soluble proteins were fractionated by anion exchange chromatography, and each fraction was assayed for the ability to refold preaggregated FFL either alone (○) or in the presence of human Hsp70 and Hdj2 (●). An equal volume of each fraction was separated by SDS−PAGE and analyzed by Western blot with anti-Hsp104 antibody. (A) Extract from PrtTAHsp104 induced for 48 h. (B) Extract from uninduced PrtTAHsp104.

supplemented with Hsp70 and Hdj2 (Figure 7A). Western blot analysis of the fractions indicated that refolding-active fractions contained Hsp104. Refolding could not be detected in fractions from cells that were not induced (Figure 7B).

DISCUSSION

In yeast, Hsp104 expression not only quantitatively enhances survival and protein refolding, but also contributes a chaperone activity that is distinct from that of most chaperones, the ability to resolubilize and refold proteins that are already aggregated. The only Hsp100 family members that are present in mammalian genomes are a mitochondrial ClpX ortholog (*30*, *31*) that functions in proteolysis with human ClpP and Skd3, an unusual Hsp100 with a single AAA module and N-terminal ankyrin repeats (*32*, *33*) that may also associate with mitochondria but whose function is unknown. Thus, in the absence of an orthologous protein, the expression of Hsp104 in mammalian cells provides an exciting opportunity to examine the relationship between the chaperone capacity of cells and the ability to withstand stress.

This study represents the first comprehensive analysis of Hsp104 chaperone function in the alien environment of the human cell and its consequences vis-à-vis survival following heat shock.

In addition to its roles in protein disaggregation and thermotolerance, the low level of Hsp104 expressed in unstressed cells is critical for the mitotic stability of yeast prions [PSI^+] (*34*), [*URE3*] (*35*), and [PIN^+] (*36*). The observation that Hsp104 influences the stability of self-seeding aggregates has generated an interest in testing the effect of Hsp104 on the physical state of intracellular protein aggregates associated with human disease (*37*−*39*). Immunostaining of inclusions in patient tissues or in animal tissue culture models of polygutamine-repeat diseases as an example contain substantial amounts of molecular chaperones, including members of the Hsp70 and Hsp40 families, as well as ubiquitin and proteasome components (*40*−*43*). Despite the indications that the components of inclusions are recognized as "misfolded" by the protein folding and degradation machineries, animal cells apparently lack the capacity to efficiently disperse them. The expectation that Hsp104 might be an exceptionally potent agent in the clearance of inclusions of misfolded proteins in human cells or contribute in a more general way to stress tolerance relies on assumptions that we have investigated herein.

The ability to form a hybrid bichaperone network with the resident chaperones is of critical relevance to the function of Hsp104 as a disaggregase in a foreign milieu. In particular, the function of bichaperone networks is subject to the specific functional interaction between the Hsp100 component and the Hsp70 system required for refolding aggregated substrates. Hsp104 functions with purified *SSA1*-encoded cytosolic Hsp70 and a DnaJ-like cochaperone Ydj1 but does not refold protein with *E. coli* DnaK, DnaJ, and GrpE (*11*). Hsp78, a mitochondrial paralog of Hsp104, can function with yeast mitochondrial homologues of DnaK, DnaJ, and GrpE (Ssc1/Mdj1/Mge1) but not with cytosolic Ssa1 and Sis1, a Hsp40-like cytosolic cochaperone (*12*). We have established, using both purified human molecular chaperones and protein extracts from transgenic cells that Hsp104 is capable of cooperating with human Hsp70 or Hsc70 and either Hdj1 or Hdj2 to refold aggregated non-native protein. Although Hsp70 was consistently more active than Hsc70 in refolding reactions, this effect was also evident in cell extracts of uninduced cells. Thus, the difference in refolding activity is not specifically dependent on the differential ability of each chaperone to functionally interact with Hsp104.

Components of the yeast Hsp104/Hsp70/Hsp40 bichaperone network likely enter nuclei where they enhance restoration of nuclear structure and function following heat shock. In yeast, electron dense aggregates formed in the nucleoplasm during heat shock are dispersed during recovery in an Hsp104-dependent fashion (*10*), and at least one nuclear activity, pre-mRNA splicing, is sensitive to heat inactivation and is restored more rapidly in wild-type yeast than in *hsp104* deletion strains (*44*, *45*). Although evidence obtained by immunoelectron microscopy indicates that Hsp104 is present in the yeast nucleus (*26*) and has a role in repair of nuclear damage, little is known about the nuclear trafficking of Hsp104 in yeast. As a first step toward addressing this issue, we have recently identified a 17 amino acid segment of Hsp104 that is capable of directing a fusion protein into the yeast nucleus and that, when mutated, prevents a GFP−Hsp104 fusion protein entering the nucleus (J. M. Tkach and J. R. Glover, unpublished observation).

In animal cells, the nuclear matrix is potentially a critical target of thermal stress (for review, see ref *46*), and in models of polyglutamine expansion disease, aggregates frequently take the form of nuclear inclusions. Because the Hsp104 hexamer is far too large (~600 kDa) to enter the nucleus without the intervention of nuclear transport factors, establishing that Hsp104 indeed shuttles across the nuclear envelope of mammalian cells is an important finding that suggests the interaction between Hsp104 and nucleocytoplasmic trafficking factors is conserved between yeast and human cells. In contrast to Hsp70, which enhances the reactivation of thermally inactivated FFL primarily in the cytoplasm (*25*), Hsp104 contributes to the chaperone capacity of both the cytoplasm and nucleoplasm.

The dual role of molecular chaperones as mediators of cellular repair in response to stress and as suppressors of apoptosis is an intriguing phenomenon. In addition to its role in the prevention of irreversible protein aggregation, Hsp70 binds to apoptosis inducing factor-1 (AIF-1) blocking AIF-induced chromatin condensation (*47*). Hsp70 also binds to apoptosis protease activating factor (Apaf-1), hindering the recruitment of procaspase-9 to the apoptosome (*48*, *49*), a high molecular weight aggregate of Apaf-1 and cytochrome *c* released from mitochondria. Hsp70 also interferes with the apoptotic pathway at a stage before the release of cytochrome *c* from mitochondria (*21*); overexpression of Hsp70 attenuates the phosphorylation/activation state of JNK by preventing heat inactivation of a JNK phosphatase (*21*, *50*) and by binding directly to JNK and preventing its phosphorylation by the mitogen-activated protein kinase SEK-1 (*51*).

In heat-stressed mammalian cells, it is possible that the presence of misfolded proteins results in a reallocation of Hsp70 to fulfill its function in protein folding while simultaneously permitting the derepression of apoptotic signaling. The duration and severity of the stress, coupled with the ability of the cell to mount a sufficient defensive response, could determine whether the balance is tipped toward recovery or self-annihilation. The role of Hsp70 in regulating the apoptotic response is probably not limited to scenarios involving physical or chemical stress. In recent analyses of the impact of polyglutamine expansion on the cellular stress response (*52*, *53*), polyglutamine aggregation was associated with reduced or delayed Hsp70 expression and prolonged activation of JNK associated with inactivation of JNK phosphatase. Thus, in models of polyglutamine expansion disease, reduction of cellular toxicity by Hsp70 overexpression may have as much to do with the increased availability of Hsp70 to attenuate apoptotic signaling as with the action of Hsp70 in the management of misfolded proteins. In fact, at least one study concludes that the protection provided by Hsp70 in a model of polyglutamine expansion disease is more closely associated with inhibition of procaspase cleavage than with suppression of aggregation (*54*). These studies underscore an important caveat with respect to the interpretation of cytoprotection conferred by Hsp70.

The work described herein establishes that a similar caveat may apply to the expression of Hsp104. Hsp104 also inhibited proapoptotic signaling in human cells but in a manner that was distinct from that of Hsp70 overexpression.

Because JNK activation was unperturbed by Hsp104 expression, we can conclude that Hsp104 does not substitute for Hsp70 in preventing the heat inactivation of JNK phosphatase (*21*, *50*) or preventing phosphorylation of JNK by SEK-1 (*51*). Instead, it is possible that accelerated repair of misfolded proteins in the presence of Hsp104 may permit Hsp70 to resume its antiapoptotic role more rapidly following heat stress. Alternatively, Hsp104 contributes not only to the refolding capacity of cells, but also blocks the apoptotic signaling pathway at an intermediate step upstream of procaspase-3 cleavage either alone or in concert with Hsp70. Although biochemical analysis demonstrates that protein rescue by bichaperone networks depends on a synergistic interaction between chaperone systems, we cannot conclude from these experiments that the disaggregation and refolding of thermally aggregated proteins contribute directly to stress tolerance. Indeed, whereas Hsp104 confers a 100−1000-fold enhancement of survival in yeast exposed to acute, severe heat shock where heat-induced aggregation may be severe, its effect on thermal protection in our model system, featuring relatively milder heat-shock conditions, is modest. Because the effect of Hsp104 expression on apoptotic signaling appears to be distinct from that of Hsp70 overexpression, further analysis will be required to pinpoint the role of Hsp104 in the inhibition of stress-induced apoptosis. This effort could provide insight into the complex linkage between molecular chaperones, protein misfolding, and apoptosis.

Finally, we examined fractionated PEER cell extracts to investigate the possibility that an endogenous factor may be able to substitute for Hsp104 in refolding aggregated FFL with or without supplemental human Hsp70 and Hdj2. Two indications raise this possibility. First, in contrast to yeast where in vivo reactivation of thermally denatured model substrates is almost entirely dependent on Hsp104 (ref *10* and J. M. Tkach and J. R. Glover, manuscript submitted), the reactivation of FFL in PEER (parts A and B of Figure 4) and other mammalian cells (*43*, *53*) is substantial even in the absence of Hsp104. Second, addition of Hsc70 or Hsp70 to unfractionated lysates results in enhanced refolding of aggregated FFL in vitro (Figure 5D), suggesting that Hsc/p70 abundance may be limiting for the function of an endogenous disaggregase. The biochemical approach that we have taken is highly sensitive and can, in principle, detect disaggregating activity with high fidelity as demonstrated by the robust detection of Hsp104 in fractionated extracts from induced cells. In contrast, no activity capable of refolding aggregated protein was detected in fractionated extracts of uninduced cells. This represents the first analysis of aggregated protein refolding in fractionated extracts, and we cannot exclude the possibility that disaggregation in unfractionated lysates is mediated by several components working in concert, which do not cofractionate as a complex, or that a single disaggregase that is not particularly abundant in PEER cells could substitute for Hsp104 in other cells or tissues.

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