Comparison of Intra-organellar Chaperone Capacity for Dealing with Stress-induced Protein Unfolding

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Molecular chaperones are essential for cells to prevent that partially unfolded proteins form non-functional, toxic aggregates. This requirement is increased when cells experience protein unfolding stresses and such could affect all compartments in the eukaryotic cell. Whether all organelles are equipped with comparable chaperone capacities is largely unknown, mainly due to the lack of suitable reporters that allow such a comparison. Here we describe the development of fluorescent luciferase reporters that are sorted to various cellular locations (nucleus, cytoplasm, endoplasmic reticulum, and peroxisomes) and that differ minimally in their intrinsic thermal stability properties. When heating living cells, the rate of inactivation was most rapid for the nuclear-targeted luciferase, indicating that the nucleus is the most sensitive organelle toward heat-induced denaturing stress. Post-heat re-activation, however, occurred at equal kinetics irrespective of luciferase localization. Also, induction of thermostolerance by a priming heat treatment, that coordinate up-regulates all heat-inducible chaperones, resulted in a transient heat resistance of the luciferase in all organelles in a comparable manner. Overexpression of the main heat-inducible Hsp70 family member, HspA1A, protected only the cytosolic and nuclear, but not the other luciferases. Together, our data suggest that in each compartment investigated, including the peroxisome in which so far no chaperones could be detected, chaperone machines are present and can be induced with activities similar to those present in the cytosolic/nuclear compartment.

Heat shock proteins (HSP) are a subclass of essential molecular chaperones found in all organisms studied to date. Molecular chaperones assist in many fundamental biological processes such as the folding and assembly of newly synthesized proteins, transport of proteins across membranes, activation of signal transduction routes, and folding or degradation of misfolded and aggregated proteins (1–3). After the sequencing of the human genome, it became apparent that over 60 different HSP genes exist that encode for proteins with homology to one of the known classes of HSP families. Considerable progress has been made in elucidating the function of a few representative members within each subclass of chaperones. However, the precise function of most chaperones paralogs within a specific family remains unknown. Within each subclass of chaperones, members are found to be sorted to various subcellular localizations. For example, Hsp70 family members have been found that are located in the cytosol and the nucleus (Hsp70/HSPA1A/B, Hsc70/HSPA8), mitochondria (Mortalin/HSPA9B), and the ER (Bip/HSP5) (4–7). Likewise, Hsp40/DNAJ proteins have been found in the cytosol and nucleus (Dj-1/DNAJB1, Dj-2/DNAJA1), mitochondria (tid1/DNAJA3), and the ER (ERdj1/DNAJC1, ERdj2/SEC63, ERdj3/DNAJB11, ERdj4/DNAJ9, and ERdj5/DNAJC10) (8–14). So, it seems reasonable to assume that different HSP genes were duplicated during evolution to facilitate the same function of a specific HSP within a different cellular compartment. Interestingly, mammalian peroxisomes might be an exception with respect to the presence of organelle-specific chaperones. Although the Hsp70 machine is clearly involved in sorting of proteins to the peroxisomal matrix (15, 16), no classical chaperones have been found inside the peroxisomes of animal cells through proteomic analysis (17, 18). The lack of peroxisomal chaperones in animal cells seems surprising as peroxisomes consume high levels of oxygen and hence are likely to experience high levels of oxidative stress (19, 20).

Thermal stress as well as many other stresses that alter protein conformation are likely to affect proteins in many, if not all cellular compartments. Yet, biophysical (21) and biochemical insolubilization assays (22) have indicated that protein denaturation is particularly rapid for proteins in the nucleus, and it has been suggested that nuclear damage following protein unfolding stresses is most critical to stress-induced cell death (23). This is accentuated by the finding that many Hsps migrate to the nucleus upon stresses like heat shock (4, 24) (25) indicating the need for (extra) chaperone activity in this compartment. However, to date, it is unclear whether compartmental differences in thermal sensitivity are merely related to the intrinsic thermal properties of compartment-specific proteins or to different capacities of compartment-specific chaperones to deal with unfolded proteins.

Besides intrinsic heat sensitivity of (naive) cells that may depend on the ratio between constitutive expression of chaperones and thermal lability of endogenous proteins in different
compartments, cells can also acquire a transient state of resistance to heat if pre-treated with mild priming heat doses. This induction of resistance against subsequent (severe) heat stresses (mostly referred to as thermotolerance) can induce cross-resistance to many other stressors that induce protein unfolding (3, 26). The ability to survive these stresses by thermotolerant cells was paralleled by up-regulation of heat shock proteins (27). Several members of the family of the Hsp seem to be coordinately up-regulated as a result of heat shock via an autoregulatory mechanism involving HSF-1 activation that drives heat shock protein-gene activation with heat shock consensus elements in the promoter of many heat shock protein encoding genes. The ability to survive stress in thermotolerant cells indeed was paralleled by resistance to overall protein denaturation (28) and clearly also to a better recovery/repair of protein damage in the cell nucleus and cytosol (22, 25, 29). Whether a similar thermotolerance development also holds true for other compartments remains to be elucidated.

Firefly luciferase protein has proven to be an excellent reporter protein for measuring heat unfolding and chaperone activity; it is heat labile, easy to quantify, and has no background activity in mammalian cells (30, 31). Using luciferase as a reporter, we found that its targeting to the nucleus enhanced its activity in mammalian cells (30, 31). Using luciferase as a reporter, we found that targeting to the nucleus enhanced its activity in mammalian cells (30, 31).

### EXPERIMENTAL PROCEDURES

#### Cell Culture and Transfections—Flp-In T-Rex HEK293 cells stably expressing the Tet repressor were obtained from Invitrogen and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 5 µg/ml Blasticidin (Sigma), and 100 µg/ml Zeocin (Invitrogen). Cells were stably transected using Lipofectamine (Invitrogen) and stable clones were selected with 0.5 mg/ml Geneticin (Invitrogen). To minimize clonal artifacts, clones were pooled and moderate levels of eGFP expressing cells were isolated from the population using fluorescence-activated cell sorter sorting. Transient transfections were performed using Lipofectamine.

#### Plasmids, Markers of Different Cellular Compartments—pDsRed2-ER, pDsRed2-Mito, and pDsRed2-peroxi were purchased from Clontech Laboratories. The plasmid pCDNA3-H2B-mRFPruby encoding Histon H2B fused to the human codon optimized monomeric red fluorescent protein ruby (mRFPruby) (32) was created by isolating the H2B-mRFPruby fusion from pAC5.1-H2B-mRFPruby (32) after cutting at the codon optimized monomeric red fluorescent protein ruby (mRFPruby) (32) was created by isolating the H2B-mRFPruby fusion from pAC5.1-H2B-mRFPruby (32) after cutting at the

### Constitutive Expressing Luciferase Constructs—The human codon optimized luciferase was amplified from pGL3 (Promega) using the primers luc-f and luc-r (Table 1) and cloned between the PstI and BamHI sites of peGFP-N1 (Clontech Laboratories) giving Cyt/Nuc-superluc-eGFP. The Cyt-superluc-eGFP construct was constructed by ligating the annealed nuclear export signal encoding oligos HTLV2-nes-f and HTLV2-nes-r between the Nhel and EcoRI sites of Cyt/Nuc-superluc-
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eGFP. A human codon optimized version of nuclear luciferase was constructed as follows. The NarI fragment harboring the SV40 large T antigen nuclear localization signal plus the backbone of the pcGFP-N2 vector was isolated from a non-human codon optimized luciferase in eGFP-N2 (Clontech Laboratories) as described before (29). This fragment was ligated to the NarI fragment of the human codon optimized Cyt/Nuc-superluc-eGFP vector yielding Nuc-superluc-eGFP. Cox8 Mit-superluc-eGFP was constructed by isolation of the luciferase encoding BglII-NotI fragment from Cyt/Nuc-superluc-eGFP and ligation in it the BamHI-NotI cut pDsRed2-Mito vector. DmHsp22 Mito-superluc-eGFP was constructed by PCR amplification of the first 81 nucleotides corresponding to the first 27 amino acids of the Drosophila melanogaster Hsp22 gene (34). Primers used were: Hsp22-mito-f and Hsp22-mito-r. After amplification, the product was cut with HindIII and EcoRI and ligated between the HindIII and EcoRI sites of the Cyt/Nuc-superluc-eGFP construct. For the ER-superluc-eGFP, the KDEL retrieval sequence was added by PCR amplification with Cyt/Nuc-superluc-eGFP as a template. Primers used were cmv-f and ER-retrieval. After amplification, the product was cleaved with EcoRI and NotI and ligated in an EcoRI-NotI cut Cyt/Nuc-superluc-eGFP vector, thereby replacing the luciferase-eGFP gene for a KDEL-encoding luciferase gene yielding the superluc-eGFP-KDEL vector. Subsequently, the calnexin pre-sequence was added by ligating the annealed oligos Calnexin-sense and Calnexin-antisense between the NheI and EcoRI sites of the superluc-eGFP-KDEL vector. The Per-superluc-eGFP vector was constructed by adding the peroxisomal targeting sequence SKL by PCR. Primers used were: cmv-f and peroxi-r. The PCR product was cleaved with EcoRI and NotI and ligated in the EcoRI-NotI cleaved Cyt/Nuc-superluc-eGFP vector, thereby replacing the luciferase-EGLP gene for a luciferase-eGFP gene encoding a C-terminal SKL sequence.

Tetracycline-inducible Luciferase Constructs—Tetracycline inducible Cyt/Nuc-superluc-eGFP and Nuc-superluc-eGFP were constructed by subcloning the HindIII-NotI luciferase-eGFP fragment from either Cyt/Nuc-superluc-eGFP or Nuc-superluc-eGFP between the HindIII and NotI sites of pcDNA5/FRT/TO (Invitrogen). To generate the tetracycline-inducible ER-superluc-eGFP construct, the ER-superluc-eGFP fragment was PCR amplified using primers FRT/TO ER-superluc-eGFP-f and ER-retrieval. Subsequently, the PCR fragment was cleaved with HindIII and NotI and cloned between the HindIII and NotI sites of pcDNA5/FRT/TO.

Tetracycline-inducible Hsp70 Constructs—Sequences corresponding to the V5 tag were introduced to discriminate endogenously expressed Hsp70 from transfected exogenous luciferase. The V5 tag harboring a Kozak consensus ATG initiation codon and lacking a stop codon was cloned by ligating the annealed oligos V5-sense and V5-antisense between the HindIII and BamHI sites of the pcDNA5/FRT/TO vector yielding pcDNA5/FRT/TO V5. Subsequently, the coding sequence of the HSP70 gene was amplified using primers HspA1A-f and HspA1A-r and the product was cloned between the BamHI and NotI sites of pcDNA5/FRT/TO V5 yielding pcDNA5/FRT/TO V5 HspA1A. The multicloning site of the pcDNA5/FRT/TO V5 HspA1A vector was modified to clone a nuclear localization signal. The V5 HspA1A sequence was amplified using the V5-HspA1A-f and V5-HspA1A-r primers and the product was cloned between the EcoRV and NotI sites of the pcDNA5/FRT/TO vector. Finally, the SV40 large T antigen nuclear localization signal harboring a Kozak consensus ATG initiation codon and lacking a stop codon was cloned by ligating the annealed oligos Nuc-V5-HspA1A-sense and Nuc-V5-HspA1A-antisense between the HindIII and EcoRV sites of the modified pcDNA5/FRT/TO V5 HspA1A vector yielding pcDNA5/FRT/TO Nuc-V5-HspA1A. All sequence modifications were sequence verified and the expression was verified by Western blotting.

Luciferase Measurements—Cell lysis and luciferase activity measurements were done as previously described (22). Luciferase activity after treatments were expressed relative to the activity in unheated control cells (=100%). Error bars in plots represent standard deviations. In the case of a single experiment, the standard deviation was calculated according to the rules of error propagation. In the case of multiple experiments, standard deviations were calculated directly from the differences in the percentages of luciferase activity compared with an unheated control.

Western Blotting—Standard Western blot procedures were used as previously described (22). Antibodies used were: eeGFP J-L8 (Clontech), αV5 (Invitrogen), αHspA1A/HspA1B SPA810 (Stressgen, Ann Arbor, MI), αHspA8 SPA815 (Stressgen), αDnaB1 SPA400 (Stressgen), and αGapd (RDI Research Diagnostics, Concord, CA). Western blots were quantified using the Gel Pro Analyzer software package (Media Cybernetics, Inc., Bethesda, MD).

Immunolabeling—Cells were plated on poly-L-lysine (0.001%, w/v), Sigma) coated coverslips. The next day, cells were transfected with 1 μg of the pcDNA5/FRT/TO V5-HspA1A plasmid or Nuc-V5 HspA1A plasmid. The gene expression was induced with a final concentration of 1 μg/ml tetracycline. After one extra day, cells were treated as described previously (35). The V5 antibody was used at a concentration of 1:200. The CY-5 antibody was purchased from Jackson ImmunoResearch (West Grove, PA) and used at a concentration of 1:200.

Confocal Microscopy—For confocal micrographs, cells were grown on coated coverslips and fixed with 3.7% formaldehyde. To visualize nuclei, cells were stained 10 min with 0.2 μg/ml 4′,6-diamidino-2-phenylindole. Coverslips were mounted with 1:200. The CY-5 antibody was purchased from Jackson ImmunoResearch (West Grove, PA) and used at a concentration of 1:200.

Reverse Transcriptase-PCR—Cells were treated for 30 min at 45 or 37 °C and total RNA was isolated after 0, 3, 6, 12, and 24 h of incubation at 37 °C. Total RNA was isolated using the Absolutely RNA isolation kit (Stratagene). Subsequently, 1 μg of total RNA was transcribed in first strand cDNA using Moloney...
murine leukemia virus reverse transcriptase (Invitrogen). The cDNA synthesis was performed with oligo(dT)_{12-18} (Invitrogen). Thereafter, the cDNA was amplified with Platinum Blue PCR Supermix (Invitrogen) using the following conditions: 2 min at 94 °C, 25 or 30 cycles (GAPDH, HSPA1A, and DNAJB1) for 15 s at 94 °C, and 15 s at 54 °C and 30 s at 72 °C. Primers and sorted the moderate eGFP expressing fraction by min at 94 °C, 25 or 30 cycles (GAPDH, HSPA1A, and DNAJB1) for 15 s at 94 °C, and 15 s at 54 °C and 30 s at 72 °C. Primers

### RESULTS

Subcellular Targeting of Luciferase—To be able to compare intra-organellar chaperone capacities for dealing with stress-induced protein unfolding, intra-organellar reporters are required that have similar and rapid intrinsic denaturation properties and that can be simultaneously followed by fluorescence microscopic techniques for proper localization studies. Fusion of luciferase to eGFP is expected to result in such a desired recombinant protein. Luciferase is rapidly denatured at mild to high heat shock regimes of 40–45 °C (22). In contrast, eGFP forms a very heat stable globular protein that is fluorescent at temperatures exceeding 55 °C (36). To target luciferase to various organelles, only experimentally verified and well described targeting sequences were selected. We created recombinant luciferase molecules with localization sequences to target luciferase to the cytosol, nucleus, mitochondria, endoplasmic reticulum, and peroxisomes (Table 2). First, we used transient expression to verify the localization of the recombinant proteins in HEK293 cells. However, we found that under these conditions the localization of the peroxisomal luciferase reporter was primarily cytosolic with only a minority of the cells in the population showing the typical “peroxisomal dot-like” expression (data not shown). This phenomenon has been reported before for transient expression protocols (37). To resolve this issue, we generated stable cell lines for all constructs and sorted the moderate eGFP expressing fraction by fluorescence-activated cell sorter. Subsequently, we examined the localization of the luciferase reporters in the stable clones. As can be seen in Fig. 1A, the luciferase reporter without any fused localization signal was found in both the cytosol and the nucleus. To obtain an exclusive cytosolic luciferase protein, we exploited a well described nuclear export sequence derived from the human T-cell leukemia virus type 2 Tax protein (38) and fused it to the N terminus of luciferase (Table 2). Indeed, this luciferase protein was found exclusively in the cytosol (Fig. 1B). Likewise, luciferase targeted to the nucleus using the SV40 nuclear localization signal (39) perfectly co-localized with a mRFP-ruby-tagged Histob 2 subunit (Fig. 1C). The ER-localized luciferase was designed using the calreticulin targeting sequence (40) fused to the N terminus of luciferase and with the KDEL retrieval sequence (41) fused to its extreme C terminus. It was found that this protein co-localized with a commercial ER-targeted Dsred2 protein (Fig. 1E). Stable expression of luciferase targeted to the peroxisomes by the addition of the peroxisomal targeting signal SKL (42) revealed the typical peroxisomal dot-like pattern that colocalized with a commercial peroxisomal targeted Dsred2 construct (Fig. 1F). Indeed, stable expression of peroxisomal luciferase was not associated with the mis-localization as observed with transient expressions. Unfortunately, we failed to properly target luciferase to mitochondria in HEK293 cells. Targeting luciferase using the cytochrome c oxidase subunit 8 (Cox8) pre-sequence (43, 44) did not result in a similar expression as the mitochondrial Dsred2 marker, which also harbors a Cox8 targeting sequence. For this luciferase construct, the majority of the luciferase remained in the cytosol (data not shown). As an alternative, we used a well studied mitochondrial localization signal from the *D. melanogaster* HSP22 gene. DmHsp22 is found to be localized in mitochondria upon introduction in human cells (34). Although a significant number of cells showed that this recombinant protein did co-localize with the Cox8 Dsred2 protein (~50%), we found that a large fraction of luciferase was still found in the cytosol (Fig. 1D). Further optimization efforts in transient expression settings did not improve the specific localization. Therefore we eliminated the use of this reporter construct in functional assays from the rest of the study.

Characteristics of Subcellular Targeted Luciferase—Next, we investigated the expression levels of the recombinant luciferase levels in the different stable cell lines. As can be seen in Fig. 2A, a large variation in both activity (measured as RLU, Fig. 2A) and total expression level (Fig. 2B) was found for the differently targeted luciferases. The lowest activity and levels were found for the ER-targeted luciferase and the highest activity and levels were found in the luciferase lacking any targeting signal (Cyt/Nuc). The activity and expression levels for the other 3 luciferases (Cyt, Nuc, and Per) were comparable. The activity of luciferase (Fig. 2A) correlated with the amount found on Western blots (Fig. 2B), suggesting that the activity of the protein was not altered dramatically by the addition of the target sequence or by putative post-translational modifications in any of the compartments. As similar data were found for transient transfections, the variation in luciferase levels are most likely due to differences in protein synthesis efficiencies for luciferase in the cytosol versus the ER and not caused by technical issues such as the copy number or the genomic context of the integrated plasmids.

### Comparison of Intra-organellar Protein Refolding

![Comparison of Intra-organellar Protein Refolding](http://www.jbc.org/)

### Table 2

Luciferase constructs used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>N-Extension</th>
<th>C-Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol/Nucleus (Cyt/Nuc)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cytosol (Cyt)</td>
<td>HTLV2-NES</td>
<td>None</td>
</tr>
<tr>
<td>Nucleus (Nuc)</td>
<td>SV40 Large T NLS</td>
<td>None</td>
</tr>
<tr>
<td>Mitochondria (Mit)</td>
<td>Cox8 and DmHSP22 pre-seq</td>
<td>None</td>
</tr>
<tr>
<td>ER (Endoplasmic Reticulum)</td>
<td>Calnexin pre-seq</td>
<td>KDEL</td>
</tr>
<tr>
<td>Peroxisomes (Per)</td>
<td>None</td>
<td>SKL</td>
</tr>
</tbody>
</table>

The abbreviations used in the table are: HTLV-2, human T-cell leukemia virus type 2; NES, nuclear export sequence; SV40, simian virus 40; NLS, nuclear localization sequence; Cox8, cytochrome c oxidase subunit 8; DmHsp22 pre-seq, *Drosophila melanogaster* heat shock protein 22 pre-sequence.
Hsc70 (HSPA8), Hsp70 (HSPA1A), and HSP40 (DNAJB1) were found not to differ significantly between the different stable cell lines (Fig. 2B) or from the parental cell line (supplemental Fig. 1A) indicating that overexpression of none of the recombinant luciferase transgenes induced a general stress response.

The heat sensitivity of a protein is both dependent on extrinsic factors (defined by the environment of the organelle where the protein is located) and intrinsic factors (the globular fold defined by the primary amino acid sequence). To study the differences in heat sensitivity of organelles, it was therefore important to first test whether the modification of luciferase by the fusion of a sorting sequence had changed the intrinsic heat sensitivity of the protein. Here, we lysed cells expressing the different luciferase proteins, and subsequently diluted the samples to yield similar RLU values (i.e. to equalize its concentration) and heated the lysates. So, all luciferases were now in an equal environment before heating. As found before (22), luciferase in cell lysates is even more sensitive to thermal denaturation than when present within its cellular environment. Therefore denaturation kinetics had to be monitored at 38 °C. The cytosolic luciferase harboring a nuclear export signal was slightly more heat sensitive compared with the other luciferases that showed similar inactivation kinetics (Fig. 2C) that were equal to that of the non-tagged luciferase-eGFP (data not shown).

The experiment was repeated with similar volumes of the lysate instead of similar RLU values with similar results (supplemental Fig. 1B). In summary, only the luciferase harboring a nuclear export signal showed slight enhanced heat sensitivity, suggesting that the NES had some minor destabilizing effects on the protein making it a bit more denaturation-prone.

The library of organellar luciferase constructs was intended to study differences in compartment-specific protein unfolding and refolding. However, the luciferase assay is unable to discriminate between heat-induced luciferase inactivation and the possible effect of heat shock on the degradation of luciferase. Therefore, we tested whether heat shock induced the degradation of luciferase. As shown in Fig. 2D, heat shock at 45 °C followed by up to 3 h of recovery at 37 °C did not induce significant degradation of any of the luciferases. So, within this time period, we can use activity measurements to compare the compartmental chaperone activities.

We also tested whether the application of a heat shock to intact cells resulted in changes in the localization of the different luciferases as could theoretically be caused by heat-induced changes in cell morphology (46) that might compli-
cate the interpretations of results. Indeed, heating caused the cells to partially round up. In addition, the accumulation of aggregate-like foci of the various luciferases was seen but the localization of the various luciferases remained mostly unaltered (Fig. 3A). Only a fraction of the cytosolic luciferase was seen in or collapsed on the nucleus after heat shock, but this effect was largely reversed 1 h after heating (Fig. 3A).

Possibly, components of the nuclear export machinery are unfolded by the applied heat stress leading to passive diffusion of the cytosolic luciferase to the nucleus. To test in how far reliable compartment-related questions regarding the cytosolic luciferase could be addressed, we followed its behavior in time at 45 °C. Up to 20 min, no significant nuclear accumulation of the cytosolic luciferase was seen (Fig. 3B) indicating that for analysis of its compartmental dependent responses, data on inactivation kinetics up to 20 min of heating are reliable.

Heat Sensitivity of Luciferase in Situ—Having performed all these control experiments, we next asked the question whether the different subcellular organelles differ in their response to heat shock-mediated effects on an identical substrate in terms of its intrinsic thermostability. Cells stably expressing the different luciferase constructs were heated at 45 °C for various times and subsequently lysed to measure the remaining luciferase activity. As shown in Fig. 4A, luciferase was most rapidly inactivated when present in the cell nucleus, confirming our previous data that implies that the nuclear compartment is very heat sensitive (22). Inactivation kinetics in all other compartments were equal, implying that inactivation in the cytosolic compartment may in fact be a bit less rapid because the NES-luciferase had a slightly higher intrinsic heat sensitivity (Fig. 2C). To exclude differences in expression levels between the luciferase (Fig. 2A) as a confounder for our measurement on luciferase inactivation rates, we expressed all luciferase to equal RLU levels using transient transfection with the various luciferase encoding constructs (except the peroxisomal that mislocalizes under such conditions) under the control of a tetracycline-inducible system. At these equal levels of expression (equal RLU) the inactivation rates showed comparable sensitivity patterns (supplemental Fig. 1C) as those presented in Fig. 4A, indicating that the compartmental sensitivity differences were unrelated to differences in expression levels of the different luciferases.

We then performed a similar experiment albeit now studying the organellar heat sensitivity dependent on heating temperature. Due to the fact that cytosolic luciferase accumulates slightly in/at the nucleus after 20 min heating (Fig. 3B), the heating time was set to 15 min. Again the nucleus was found to be a very heat-sensitive organelle except at the mildest heating temperatures (39 °C), at which the ER-luciferase declined most rapidly (Fig. 4B). Also in this case, differences in expression levels were unrelated to the temperature-dependent organellar heat sensitivity (supplemental Fig. 1D).

It was previously shown that both in the nucleus and cytosol significant fractions of heat-inactivated luciferase can be reactivated in time after heating, in a manner that depends on the action of molecular chaperones (25, 35). To test whether this is also true for the ER and peroxisomes, cells expressing the vari-

FIGURE 2. Biochemical characterization of organelle-specific luciferases. A, luciferase activity in the stable luciferase expressing cell lines. Luciferase activity was corrected for protein content. Data are the mean ± S.D. of four replicates. B, expression levels of subcellular sorted luciferase and various HSPs analyzed by Western blot. Samples were run on the same gel but rearranged for consistency and clarity. C, in vitro denaturation of luciferase. Cells were lysed, and the cell extracts were heated at 38 °C and luciferase was measured. Luciferase activity was logarithmically plotted relative to the activity of an unheated control. Data are the mean ± S.D. from a single experiment with 3 replicates. D, Western blot analysis of organelle-sorted luciferase levels under 37 °C control (control) conditions, after 30 min of 45 °C heat shock (HS) and 1, 2, or 3 h of recovery at 37 °C (rec). Cycloheximide was used to block de novo synthesis of luciferase. For clarity, a longer exposure blot of the ER-targeted luciferase was used.
ous luciferases were heated at 45 °C for 30 min and subse-
quently placed at 37 °C to allow time for refolding. Cyclohexi-
mide was added to prevent de novo synthesis of luciferase. As
can be seen in Fig. 4C, reactivation of luciferase occurred with
similar kinetics within the first hour in all of the organelles
tested, whereas for the subsequent 2 h the nucleus showed a
slightly faster refolding compared with the other organelles.
Because the nuclear-luciferase was hypersensitive, the cells
expressing this luciferase were also heated at 43 °C instead of
45 °C to yield a similar fraction of inactivation for all luciferases
immediately after heat shock (Fig. 4D). Again, refolding of lucif-
erase was evident at roughly similar rates in all organelles tested
(Fig. 4D).

Acquisition of Thermotolerance—Heat pre-conditioning can
induce a transient resistance toward a second heat-induced
stress, a phenomenon referred to as thermotolerance. Thermo-
tolerance is closely linked to the elevated expression of heat
shock proteins that can lead to protection against toxic protein
damage (27). We previously showed that nuclear and cytosolic
resident luciferase are protected in pre-conditioned, thermo-
tolerant cells (22), consistent with the fact that the main heat-
inducible chaperones are localized in or reallocating to these
compartments upon heating. Whether comparable build-up of
thermotolerance is also seen for the ER or peroxisomes is yet
unknown.

First, different heat pretreatments were used to search for an
optimal thermotolerance inducing protocol. The extent of
refolding of cytosolic luciferase up to 1 h after a second heat
treatment of 45 °C for 30 min was used as a readout. Fig. 5A
shows that the highest heat pre-conditioning dose of 30 min at
45 °C induced the highest degree of thermotolerance. As can be
seen from Fig. 5B, both the cytosolic/nuclear Hsp70 (HSPA1A)
and Hsp40 (DNAJB1) transcription was strongly induced under
these conditions and the highest level of mRNA expression was
found around 6–12 h post-heat shock. Using this protocol,
thermotolerance was found for luciferases in all compartments
tested. Moreover, with the exception of the ER that showed low
levels of thermotolerance, the different compartments show
strikingly similar levels of thermotolerance all peaking around
12 h post-heat shock (Fig. 5C). Thus all tested organelles show
an adaptive response to the pre-heat shock applied that resulted
in elevated chaperone activity.

Compartment-specific Chaperones—The above experiments
suggested that heat-induced factors can result in thermotoler-
ance in each organelle tested. Our previous work has shown
that the HspA1A chaperone machine is highly crucial for the
handling of heat-denatured luciferase in the cytosol/nucleus
(25). Whereas heat-inducible family members of this Hsp70
machine are also found in the ER (48) and thus could be respon-
sible for thermotolerance in this compartment, peroxisomal
chaperones have not been found so far in animal cells through
proteomic analysis (17, 18). It is conceivable that components
of the cytosolic Hsp70 machine migrate to the peroxisomes
upon heating but this was never demonstrated. Alternatively, a
fraction of peroxisomal luciferase may still be cytoplasmic and
it is this fraction that is protected in the pre-conditioned cells.
Although no cytoplasmic contamination of the peroxisomal
luciferase was detected in our immunohistochemical analysis
(Fig. 1) this analysis may be too insensitive to exclude such a
possibility. To address these possibilities, we therefore con-
structed a V5-tagged version of HspA1A to distinguish the
transfected Hsp70 from the endogenously expressed HspA1A.
In addition, we constructed a nuclear targeted HspA1A protein
using the SV40 large T nuclear localization signal. As shown in
Fig. 6A, the V5-tagged HspA1A protein is located in the cytosol
under non-heat shock conditions. In contrast, the nuclear-tar-
gated V5-tagged HspA1A was exclusively found in the nucleus.
Surprisingly, targeting HspA1A to the peroxisomes or the ER
failed for unknown reasons (data not shown).
Expression of untagged HspA1A or the V5-tagged HspA1A (Fig. 6B) resulted in protection of both the cytoplasmic and nuclear luciferases consistent with the notion that HspA1a is cytosolic under normal conditions but migrates rapidly in the nucleus under heat stress conditions (49). This also demonstrates that fusion of the V5 tag did not interfere with HspA1A function. For the nuclear-targeted HspA1a, no effects on refolding of the cytosolic luciferase were found while its expression resulted in substantial enhancement of luciferase refolding inside the nucleus (Fig. 6C). Expression of neither one of these HspA1A versions resulted in protective effects on the ER or peroxisomal-localized luciferases (Fig. 6C). These data demonstrate that the luciferase sorting is highly specific. Second, it can be concluded that HspA1A does not contribute to the enhanced refolding that was observed in peroxisomes (or ER) under thermostolerance conditions and thus makes it unlikely that this thermostolerance in the peroxisomes was due to effects on a fraction of peroxisomal luciferase that was misslocalized (i.e. cytosolic).

**DISCUSSION**

In this report we have compared chaperone function inside various eukaryotic organelles using a newly developed collection of specific reporters that target the cytosol, nucleus, ER, and peroxisomes. Using our library of organellar targeted luciferases, we found that the rate of luciferase inactivation is most rapid in the nucleus (except for very mild heating), our data show that all compartments contain systems that are equally capable of refolding heat-denatured luciferase. Also, heat pre-conditioning can induce a resistant phenotype in all these compartments reflected in enhanced luciferase refolding.

Although luciferase is an excellent model substrate and a known client of the Hsp70 machine, inter-compartmental differences in the inactivation and reactivation kinetics may not be interpreted as absolute indications for the compartmental chaperone capacities. It cannot be excluded that some compartments may contain chaperones more specifically adapted for the compartment-specific proteins and less capable of handling heat-denatured luciferase. Yet, the relative high sensitivity of the cell nucleus toward heat stress is striking and the cause for this remains unknown. A possible explanation for the enhanced sensitivity toward proteotoxic stress may lie in the fact that the nucleus has a relative low abundance of molecular chaperones. Many chaperones are primarily cytosolic, facilitating translation and post-translational quality control under normal conditions. They only migrate to the nucleus upon stress (4, 24, 25). Thus at the initiation of the heat shock, the nucleus is relatively devoid of chaperones that therefore may be more sensitive toward heat stress. Indeed, the rate of nuclear luciferase inactivation is most rapid in the initial phase of heating but parallels the denaturation pattern of the cytosolic luciferase in the later phases of heating (Fig. 4A). In line with these observations, nuclear accumulation of Hsc70 (HspA8) is found as rapid as 15 min post heating (24). It is currently not known why many chaperones are karyophobic in nature (4), but it could suggest that nuclear localization of these chaperones disturbs normal cellular function. Whatever the reason for a low abundance of chaperones in the nucleus, it likely is a major reason for the observed nuclear hypersensitivity toward thermal stress. However, we cannot exclude that chemical or physical differences between the nucleus and other organelles might play a role as well. But because the nucleus has large pores, many chemical properties are expected to be very similar to that of the cytosol. Therefore, we favor the idea that the hypersensitivity of the nuclear luciferase to heat is due to the

**FIGURE 4.** *In situ* heat sensitivity of organellar-targeted luciferases. A, time-dependent intra-organellar luciferase denaturation at 45 °C conducted in stable cell lines. Data are the mean ± S.D. of two independent experiments with 4 replicates each. B, temperature-dependent intra-organellar luciferase denaturation at 15 min conducted in stable cell lines. Data are the mean ± S.D. of two independent experiments with 4 replicates each. C, refolding of denatured luciferase after a heat dose of 45 °C for 30 min. Data are the mean ± S.D. of three independent experiments with 4 replicates each. D, refolding of denatured luciferase after a heat dose giving an approximate similar damage. Heat shock was performed at 45 °C for 30 min except for nuclear luciferase for which a heat shock regime of 43 °C for 30 min was applied. Luciferase activity was logarithmically plotted relative to the activity of an unheated control. Data are the mean ± S.D. of three independent experiments with 4 replicates each.
fact that the nucleus is relatively devoid of chaperones under non-heat shock conditions.

Another interesting finding is that the luciferase targeted to the ER is very sensitive for heat-induced inactivation at low temperatures. At higher temperatures inactivation rates of this luciferase did not differ from those in the other organelles tested. As our luciferase protein constantly shuttles from Golgi to ER due to the KDEL retrieval sequence, part of it will be present in the Golgi apparatus. For heat-denatured \(\alpha\)-lactamase targeted to the ER in yeast, it was found that refolding was only possible when the protein was located in the ER and not when it was present in the Golgi (50). This indicates that there might be no active chaperones in the Golgi and thus could explain the hypersensitivity of the ER luciferase and the corresponding biphasic inactivation curve at low temperatures (Fig. 4B) in which the luciferase present in the early

It is well established that denatured luciferase cannot refold spontaneously and needs chaperones to facilitate its refolding (51). Therefore, our finding that heat-unfolded luciferase can be refolded in every organelle tested and that in all these organelles thermostolerance could be induced, suggest that they all contain constitutive as well as heat-inducible chaperone-like systems capable of maintaining unfolded proteins in a state that prevents their irreversible aggregation and keeps them (re)folding competent. Refolding in the cytosol/nucleus is mainly dependent on the Hsp70 (HspA1A/HspA1B) machine. Although Hsp27 and Hsp90 do contribute (52, 53),\(^3\) Hsp70 is rate-limiting, especially under thermostolerance conditions (25). The ER contains a similar Hsp70 machine composed of HSPA5/BiP and various ER DnaJ members, but other heat-inducible factors may contribute as well. Consistent with an involvement of the BiP machine, reactivation of an unfolded ER targeted \(\alpha\)-lactamase in yeast was paralleled by co-immunoprecipitation of BiP and \(\alpha\)-lactamase (50).

So far, proteomic analysis on (isolated) peroxisomes did not reveal the presence of chaperones in peroxisomes of mammalian cells (17, 18). However, our data indicate that constitutive as well as heat-inducible factors must exist that stimulate the refolding of luciferase with an efficiency comparable with that found in the cytosol. So, peroxisomal chaperones exist and were they missed in proteomic analysis of mammalian peroxisomes? Or do other non-classical chaperone-like proteins perform these tasks in the peroxisomes? Interestingly, molecular chaperones of different classes have been found in the peroxisomes of plants. A Hsp70 homolog was found to be targeted to both chloroplasts and peroxisomes in \(\textit{Citrullus vulgaris}\) (54) and a DnaJ homolog was found to be attached to the glyoxisomal membrane in \(\textit{Cucumis savitus}\) (55). Also, two small heat-shock proteins, of which one was heat inducible, where found in the matrix of peroxisomes

from *Arabidopsis thaliana* (56). Thus, at least in plants classical peroxisomal chaperones do exist. Interestingly, a positive antibody staining in peroxisome-like structures for the GroEL homolog Hsp60 has been reported in rat liver cryosections (57). However, the immunogold labeling of peroxisome-like structures was low in comparison to mitochondria and there is no proteomic evidence supporting the presence of Hsp60 in peroxisomes (18). Hsp60 is a nuclear-encoded mitochondrial protein with a canonical mitochondrial import sequence. It shows no peroxisomal import sequence. Nevertheless, due to the fact that peroxisomal proteins are imported in an oligomeric and partially folded state, some chaperones might get imported along with the cargo proteins bound to Pex5p or Pex7p. But given the requirement of high levels of chaperones in other organelles, it remains unlikely that the import of a small number of chaperone proteins will contribute to the level of refolding as observed in our analysis. Furthermore, it has been shown that Hsp60 and the Hsp60 homologue GroEL can bind but not refold firefly luciferase (58, 59) making the involvement of Hsp60 in luciferase refolding in peroxisomes unlikely.

The lack of detection of classical Hsps through proteomic analysis lead us to start a bioinformatics approach to predict the subcellular localization of the classical chaperone proteins. A large degree of variability in the predicted localization of the various Hsp110, Hsp70, Hsp40 and small HSP family members was found when comparing the prediction methods CELLO, WoLFPSORT, pTarget, MultiLoc, and Proteome Analyst (supplemental Table S1). In fact, only pTarget and MultiLoc suggest that some Hsp70 members may be present in the peroxisomes, but this is not a consensus prediction, neither is it supported by experimental evidence. Clearly, the currently available bioinformatics methods lack sensitivity to be conclusive in this matter as was suggested before for predicting intracellular distributions for other proteins (47). So could it be that non-classical chaperone-like proteins are responsible for the refolding and thermotolerance we observed for luciferase residing in the peroxisomes? One possible candidate is the high abundant ATP-dependent Lon protease LonP. This mammalian peroxisomal AAA-protease is highly homologous to the mitochondrial Lon protease and has a C-terminal SKL peroxisomal import sequence (18). In prokaryotes, Lon proteases are considered molecular chaperones that perform proteolytic tasks similar to the proteasome of Archaea and eukaryotes. Hereto these proteases must unfold their substrate prior to processive peptide bond cleavage. Although Lon proteases favor unfolded or aberrant proteins, they also can cleave folded proteins and do so preferentially between hydrophobic patches located between highly charged environments at the surface of the folded protein (45). Like ATP-dependent chaperones such as Hsp70 or Hsp90, the binding of substrates to Lon proteases is regulated by ATP binding and hydrolysis cycles. Therefore, it is possible that peroxisomal Lon proteases play a role in protein refolding. However, we found that the peroxisomal Lon protease was not induced by heat shock conditions that did induce thermotolerance (supplemental Fig. S2). Consistent with these results, yeast Lon deletion mutants are still able to acquire thermotolerance (27). So, although Lon protease is an abundant chaperone-like protein found in peroxisomes, at this stage we have no evidence

![Comparison of Intra-organellar Protein Refolding](http://www.jbc.org/)

**FIGURE 6.** Effects of overexpression of HspA1A and nuclear targeted HspA1A (Nuc-HspA1A) on renaturation of organellar specific luciferases. *A*, confocal micrographs showing the immunolocalization of V5-tagged Hsp70 proteins and a nuclear targeted V5-tagged Hsp70 protein. Bar represents 10 μm. *B*, expression of V5-tagged Hsp70 proteins. *C*, cells expressing Cyt, Nuc, ER, or Per-targeted luciferase were transfected with V5-HspA1A or Nuc-V5-HspA1A, heated (30 min at 45 °C), and allowed a 1-h recovery at 37 °C after which luciferase activity was determined. Luciferase activity was plotted relative to the activity of an unheated control. Data are the mean ± S.D. of a single experiment with 4 replicates.
for LonP to function as a molecular chaperone in peroxisomes. As our data point to the existence of heat-inducible factors that facilitate in the refolding of heat-denatured luciferase, other non-classical chaperone-like proteins in the peroxisomes are possible candidates and subject for subsequent research.

Unfortunately, we were unable to succeed in targeting luciferase properly to mitochondria. Fusion with human cytochrome oxidase 8 did not result in any detectable mitochondrial localization. The reason for this targeting failure remains unclear. Possibly, the native luciferase fold interferes with the α-helical mitochondrial import presequence of cytochrome oxidase 8. Using the Drosophila Hsp22 luciferase targeting signal we found that a substantial part of the luciferase co-localized with the commercial mitochondrial localized DsRed2 marker, however, a large fraction still was seen in the cytoplasm. This does not mean that the fraction of luciferase that colocalized with mitochondria is properly folded. It could well be that only the eGFP part of the chimera protein was properly folded. Other investigators indeed found that a mitochondrial targeted luciferase was efficiently bound by Hsp60 but never reached the native state (58). Thus luciferase may not be a suitable reporter protein for measuring proteotoxic stress in mitochondria and alternatives should be developed.

In summary, we have developed novel fluorescent luciferase markers that allow to compare the action of chaperones inside subcellular organelles. These show that all compartments are equipped with a set of, sometimes yet to be identified chaperones (peroxisomes) capable of assisting in the refolding of stress-denatured substrates. Applications such as loss of function genetics will be required to investigate which chaperones inside organelles are crucial in the proteotoxic stress response. These reporters make it feasible to perform forward genetic screens to identify genes encoding proteins not previously known to be involved in the recovery from proteotoxic stress and that may provide a compartment-specific function. These experiments are especially interesting to perform with the peroxisomal localized molecular chaperase as peroxisomes from animal cells are not currently known to import any members of the well characterized molecular chaperone families. Furthermore, these markers can be used to measure whether client inactivation by other proteotoxic stresses (e.g. oxidative stress) inside the various organelles can be dealt with in a comparable manner. Finally, the markers allow to compare resistance to unfolding stresses inside the various organelles with the resistance of these compartments against protein misfolding related genetic diseases such as cystic fibrosis or polyglutamine repeat diseases like Huntington disease.

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Comparison of Intra-organellar Chaperone Capacity for Dealing with Stress-induced Protein Unfolding

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