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The chaperones Hsc70 and Hsp70 bind the protein PGK differently inside living cells

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Abstract

Differences in the physical interaction between proteins, such as binding equilibria, can provide clues to differences in function. The binding of heat shock proteins to substrate proteins in living cells is such a case. Eukaryotic cells have evolved many homologs in the Hsp70 family of heat shock proteins, each specialized for a specific function. We previously showed that Hsp70, which is up-regulated during heat shock, binds the model substrate phosphoglycerate kinase (PGK) in human cells before PGK completely unfolds. We dubbed this the 'preemptive holding' mechanism. Here we study the homolog Hsc70 (heat shock cognate protein), which is constitutively expressed in human cells even in the absence of heat shock. Recent literature has demonstrated multiple functions performed by Hsc70 in cells under normal conditions. Despite the name 'heat shock cognate,' very few studies have shown whether Hsc70 is actually involved in heat shock response. Here we corroborate the existence of an in-cell heat shock response of Hsc70. We show that Hsc70 binds PGK in human cells in a cooperative manner that correlates directly with protein thermal unfolding. This 'unfolded state holding' mechanism differs from the Hsp70 'preemptive holding' mechanism. We rationalize the difference by protein evolution: unlike Hsp70, which is upregulated to bind proteins specifically during heat shock, the finite amount of Hsc70 in cells cannot bind to still-folded proteins, or its multiple other functions would be compromised.

Keywords: Hsc70, Hsp70, heat shock protein, PGK, protein binding, in-cell binding, temperature jump, FRET

Introduction

Chaperone-protein interactions during stress are key to cell survival. Heat shock proteins such as Hsp70 play a key role.[1,2] While bacterial cells express only a single 70 kDa heat shock protein, DnaK,[3] stress response by Hsps has evolved to be highly specialized in eukaryotes. Human cells express many different homologs of the 70 kDa heat shock protein, including the heat-inducible Hsp70,[4,5] and the constitutively expressed cytoplasmic Hsp70 homolog, known as heat shock cognate protein Hsc70, which shares ~85% sequence identity with Hsp70.[6] Both proteins contain an N-terminal ATP-binding domain and a C-terminal substrate binding domain, with a similar mode of action: ATP-binding of the N-terminal domain mediates rapid sample-and-hold by the C-terminal substrate binding protein; substrates are held when ATP is hydrolyzed to ADP.[7]

Could physical binding measurements provide some clues as to the different functions of Hsp70 and Hsc70? We recently characterized the heat shock-induced binding of Hsp70 to its non-obligatory model substrate phosphoglycerate kinase (PGK) in human U-2 OS cells.[8] We showed that Hsp70 binds PGK cooperatively during heat stress in an ATP-dependent specific manner. Furthermore, we showed that Hsp70 binds PGK preemptively inside heat-shocked cells, i.e. the binding transition lies *ca.* 4 °C below the unfolding transition of PGK. We rationalized the "preemptive holdase" mechanism by increased thermal fluctuations of folded substrate that transiently expose hydrophobic patches, which are then recognized and bound by Hsp70.

Unlike Hsp70, Hsc70 performs many protein quality control functions in the cell under normal conditions.[9,10] Studies have shown that in the healthy, unstressed cell, differences appear between Hsp70 and Hsc70 function.[11] However, there is also evidence that during stress, Hsc70 and Hsp70 co-localize in various cellular compartments where they could be performing similar heat shock response functions.[12,13] Thus Hsc70 appears to assist in the heat shock response, but its behavior may differ from that of Hsp70.

In this paper we extend our previous Hsp70 in-cell study to Hsc70 to look for such a difference: we want to elucidate directly if Hsc70 assists during heat shock, but also if it may do so differently than Hsp70. Our previous research already demonstrated lack of ATP-dependent binding of Hsp70 to substrates *in vitro*,[8] so we focus our comparison on in-cell binding here. For direct comparison, we utilize the same PGK model substrate as in our Hsp70 study. We thermally denature PGK in a U-2 OS cell by using an infrared laser that heats the aqueous medium in and around the cell to induce a controlled heat shock.[8] In order to study binding in-cell, we engineered a FRET pair consisting of Hsc70 labeled with mCherry on the C-terminus (mHsc70) and a mEGFP labeled PGK at the N-

terminus (ePGK). mEGFP and mCherry have undetectable direct association in-cell.[14] Green and red intensity are monitored on a CMOS camera sensor as a function of temperature and binding is monitored by the change in FRET efficiency as a function of temperature. For all our in-cell experiments, protein concentrations are kept in the 10-15 μM range somewhat above physiological concentration of Hsc70 (ca. 6 μM according to the pax database (https://pax-db.org/).

Our results show that Hsc70 indeed binds PGK upon heat shock in the cell very much like Hsp70. However, its mechanism differs from that observed for Hsp70. Instead of binding preemptively, Hsc70 binding correlates with substrate unfolding instead of preceding unfolding by *ca.* 4 °C. This is an important functional distinction that could have far-reaching effects on how Hsp70 and Hsc70 share chaperoning tasks within the cellular milieu.

Materials and methods

Protein expression and purification Protein expression was carried out according to a previously established protocol.[8] Fusion protein sequences were cloned into pDream 2.1/MCS vector (Genscript Corp.) and used for dual expression in *E. coli* and mammalian cells unless stated otherwise. Sequences of relevant Hsc70 (HSPA8) and PGK mutants are listed in SI Table S1. Hsc70 was cloned with a C-terminal mCherry (mHsc70) and mEGFP (mEGFP-Hsc70) fusion protein with a 6×His-tag for purification. All PGK sequences were also cloned into pDream as N-terminal mEGFP fusion proteins with 6xHis-tags for purification purposes (ePGKs). PGK mutants (PGK0-PGK3) with varying stabilities were designed in-house using PCR site-directed mutagenesis. The goal of the fluorescent labeling was not to obtain a maximum FRET signal when Hsc70 and PGK interact, but to obtain enough signal while not disrupting the function of the proteins. Hsc70 was dialyzed in buffer with 100 mM Tris-HCl, 0.1 mM EDTA, 100 mM NaCl, pH 6.9 with KOH. For detailed protein expression protocols see previously published work.[8]

***In vitro* fluorimeter melts** Prior to all measurements the glycerol from the frozen stocks was removed by spin-filtration buffer exchange. Tryptophan fluorescence measurements and *in vitro* FRET binding experiments were conducted on an FP8300 spectrofluorimeter equipped with Peltier temperature control (JASCO). Tryptophan was excited at 295 nm, and emission spectra were collected from 290 to 450 nm. Samples were measured in 300 μL cuvettes at 5 μM concentrations, unless otherwise noted.

For all FRET measurements mEGFP was excited at 485 nm and emission spectra were collected from 480 to 700 nm in 300 μL cuvettes. Unfolding and aggregation of Hsc70 was monitored by the

change in FRET efficiency *vs.* temperature by melting an equimolar mixture of mHsc70 (1 μM) and mEGFP-Hsc70 (1 μM) with and without 2 mM ATP and 10 mM DTT (dithiothreitol) in K1 buffer (25 mM HEPES buffer, 50 mM KCl and 10 mM $MgCl_2$; pH 7.6 with KOH; HEPES = 2-[4-(2-**h**ydroxy**e**thyl)**p**iperazin-1-yl]**e**thane**s**ulfonic acid).

FRET binding experiments in cells Mammalian U2-OS cells were grown and cultured according to a previously published protocol.[8] Briefly cells are grown in DMEM medium (Dulbecco's Modified Eagle Medium, Corning) supplemented with 10% fetal bovine serum, 5% penicillin streptomycin (Fisher) and 5% sodium pyruvate (Fisher) (Media A). Cells were transferred to pre-cleaned glass cover slips in a 35 mm falcon dish (MatTek Corp.). They were then co-transfected with mHsc70/mHsc70K71M and the respective ePGK plasmid with lipofectamine (Fisher) in Media A without penicillin. 4 hours after transfection the media is changed to Media A and allowed to grow for 18-26 hours.

On day of experiment (18-26 hours after transfection), cells were washed and adhered to a slide using a 120 μm thick spacer (Grace Bio-Labs) for imaging. Cells were imaged in Opti-MEM medium (Fisher) supplemented with 15% fetal bovine serum. mEGFP in the cells was excited by a white LED by passing the light through a Chroma ET470/40x bandpass filter and mCherry was excited through a ET580/25x bandpass filter.[15] Both mEGFP and mCherry emission was monitored on a Lt225 (Lumenera Corp.) camera equipped with a CMOS sensor. Cells were heated using fast temperature jumps between 19-46 °C and imaged at 60 fps using LabVIEW control software (National Instruments).

A ratiometric calibration was performed by exciting various concentrations of mEGFP and mCherry and recording the room temperature intensity as a function of concentration. This *in vitro* calibration was used to determine roughly the amount of mHsc70 and ePGK in the cell at room temperature. We assumed that mHsc70 and ePGK show no binding at room temperature. Cells were selected for experiments only if they expressed 1:1 ePGK:mHsc70/mHsc70K71M, and were sufficiently bright to have a labeled protein concentration in the >10 μM range.[15]

Data analysis All data was analyzed using MATLAB (MathWorks). *In vitro* tryptophan fluorescence measurements were analyzed by monitoring wavelength peak shift. Both *in vitro* and in-cell FRET binding measurements were monitored using FRET efficiency (E_{FRET}). The E_{FRET} was calculated according to:

$$E_{FRET} = \frac{mCherry\ (acceptor)\ intensity}{mEGFP\ (donor)\ intensity + mCherry\ intensity} \quad [1]$$

In vitro intensities were calculated by measuring area under the curve between 495 to 585 nm (donor) and 585 to 700 nm (acceptor). In-cell binding was detected via FRET from mEGFP to mCherry and quantified as described previously.[8] FRET efficiency was estimated at fourteen equally spaced temperatures from 20-46 °C by interpolation and normalized to the first temperature point. The traces were then corrected for bleaching by fitting a line through the first three data points and subtracting the fitted slope. The corrected traces were then averaged.

Melting temperature (T_m) or binding temperature (T_0) were calculated using a two-state sigmoidal fit to the experimental data as described previously.[8] If binding is driven by unfolding, sigmoidal curves are also appropriate as an approximation for the bimolecular process. Data was fit according to the equations below:

$$S_i = m_i(T - T_X) + b_i\,, \qquad [2]$$

where i is either the native or substrate-chaperone unbound state (N) or the denatured or substrate-chaperone aggregated state (D), m is the slope and b is the intercept. T_X is the corresponding midpoint of the curve (T_m or T_0) with the temperature T. The total signal $S(T)$ can then be estimated as a sigmoidal function with respect to temperature T:

$$S(T) = S_N(T)f_N(T) + S_D(T)f_D(T)\,, \qquad [3]$$

where, f_N and f_D are the populations of the N and D states, respectively.

$$K_{eq} = e^{-\frac{\Delta G_{N\rightarrow D}}{RT}},$$

$$\Delta G_{N\rightarrow D} \approx \delta g_1(T - T_X), \qquad [4]$$

$$f_N = \frac{K_{eq}}{1+K_{eq}}, \text{ and } f_D = \frac{1}{1+K_{eq}}$$

Fits to equation 2 yield the melting temperature (T_m) or binding temperature (T_0). (Strictly speaking, equimolar binding as measured here is bimolecular, but eq. [4] for unimolecular reactions such as folding can still be applied for simplicity, yielding essentially the same midpoint temperature even if the shape of the bimolecular curve differs slightly from the shape of the unimolecular reaction curve.)

Competitive binding Unlike Hsp70, Hsc70 is expressed endogenously in mammalian cells (*ca.* 6 μM). Thus endogenous Hsc70 can compete with mHsc70 for PGK binding. As discussed in supplementary methods (SI p. S2), while a difference in cooperativities δg_i alone is not sufficient to shift the apparent binding temperature T_0, a difference of T_{0i} between endogenous Hsc70 and fluorescent mHsc70 can result in a shift of the apparent T_0 if the two proteins have comparable concentrations and binding affinities. Indeed, *in vitro* assays for Hsp70 show a slightly lower binding

affinity (-30%) than for mHsp70.[8] Therefore we used only bright cells in our analysis. Fig. S1 (see ref. 14) shows that the brightest 25% of cells have a GFP-labeled protein concentration in the 10-15 μM range, about 2-3 times larger than the endogenous Hsc70 concentration. In Figure 1, we compared the D/A ratio of cells expressing mHsp70 (with little unlabeled background) *vs.* Hsc70 (*ca.* 6 μM unlabeled background) because one would expect an increase of D/A if binding of ePGK to mHsc70 were reduced by competitive binding as compared to its counterpart Hsp70. The distribution of D/A for both mHsp70 and mHsc70 are comparable and the means differ by less than 10% for all cases except ePGK1 (~12%). The D/A difference for the control mEGFP is also on the same scale as for the substrate PGK. Hence, native cellular Hsc70 does not affect mHsc70 any more than mHsp70 and these proteins can be compared in-cell even if there is a slight shift in their absolute T_0.

Results

mCherry labeling moderately stabilizes Hsc70 The bacterial heat shock protein DnaK has a series of as many as four temperature-dependent transitions.[16] For human Hsp70 we found a much simpler picture: there are two transitions, one at approximately 40 °C in absence of ATP, and one at approximately 50 °C in presence of ATP. Human Hsc70, as discussed below, behaves very similarly.

Fluorescent labeling by proteins such as mCherry and mEGFP is a time-honored technique for in-cell FRET.[17,18] However, some studies have shown that labeling can perturb the labeled protein due to the fairly large size of fluorescent proteins such as mEGFP and mCherry.[19] To ensure that mCherry labeling does not significantly disrupt Hsc70 stability, we compared the stability of unlabeled wt-Hsc70 with and without ATP to mCherry-labeled Hsc70 (mHsc70) (Figure 2).

We first measured the melting temperature (T_m) of wt-Hsc70 by thermally unfolding it in a temperature-controlled fluorimeter and monitoring unfolding by tracking tryptophan peak wavelength shift. wt-Hsc70 melts with a T_m of 53 °C with ATP, and 41 °C without ATP (Figure 2A). Such stabilization upon addition of ATP has been previously demonstrated in the case of bovine Hsc70.[20]

To compare the unlabeled WT protein result with the unfolding of mCherry-labeled mHsc70, we unfolded a 50-50 mixture of donor Hsc70 (mEGFP-Hsc70) and acceptor Hsc70 (mHsc70) (Figure 2B). Unfolding in this case was measured by the FRET efficiency change (E_{FRET}) as a function of temperature. On unfolding, Hsc70 molecules aggregate which leads to an increase in E_{FRET}. Both mEGFP and mCherry are stable and do not unfold until ≥ 70 °C (SI Figure S2). mHsc70 unfolding curves fit to a T_m of 59 °C with ATP and 50 °C without ATP. A similar ~10 °C change in stability was

also observed for Hsp70 in our previous work.[8] Thus mCherry moderately stabilizes Hsc70 against unfolding in presence and absence of ATP.

We conclude that labeling with mCherry preserves ATP-dependent stability as observed previously. We further corroborated that the T_m of wt-Hsc70 is similar to mHsc70 by using temperature-controlled circular dichroism (CD) measurement in the absence of ATP (SI Figure S3). (CD measurements could not be performed in the presence of ATP because ATP absorbs UV light, and mHsc70 unfolding in the presence of ATP could not be monitored using tryptophan peak wavelength shift because the signal from the Hsc70 tryptophan is overwhelmed by the mCherry tryptophans (SI Figure S4)).

Binding of Hsc70 to PGK in-cell is cooperative and correlated with PGK unfolding PGK has previously been shown to be a non-obligatory Hsp70 substrate in-cell.[8,21] We engineered four different mutants of ePGK (ePGK0 through ePGK3) with consecutively lower melting temperatures (T_ms) to study the dependence of chaperone binding on protein unfolding. All T_ms are near or above the physiological temperature (37 °C) of a human cell line such as U-2 OS. We previously characterized the T_ms of the mutants based on tryptophan peak wavelength shift.[8] Mutant ePGK0 has a T_m (48.4±1.0 °C) well above the viable temperature range for mammalian cells and, therefore, serves as a control where little binding is expected in the temperature range tested (up to 45 °C). ePGK1 (T_m = 45.8±1.0 °C), ePGK2 (T_m = 43.3±1.0 °C) and ePGK3 (T_m = 40.5±1.0 °C), are based on different mutations (SI Table S2, CD results), and have T_ms that lie in the range where unfolding is accessible in the cell.

To study binding in-cell, both mHsc70 and ePGK were transfected into U2-OS cells using lipofectamine (see Methods). Only cells expressing roughly 1:1 mHsc70:ePGK were chosen based on a ratiometric fluorescence-intensity calibration with mCherry and mEGFP. The cells were then heated using a programmed stepped temperature jump using a 2000 nm infrared heating laser (SI Figure S5), thus thermally unfolding ePGK inside the cell. Binding was monitored by the temperature-dependent change in E_{FRET} (Figure 3). The results reported here are the average E_{FRET} change of 10 to 20 cells per experiment.

In order to distinguish between ePGK unfolding and ePGK-mHsc70 association, we refer to the protein thermal denaturation midpoint as T_m, and the association midpoint as T_0. ePGK0, due to its high T_m, showed only an onset of increased FRET and thus binding. ePGK1 showed a partial cooperative binding curve which did not go to completion and could not be accurately fitted to a

value of T_0. Both ePGK2 and ePGK3 showed fully-resolved cooperative binding curves with T_0 of 42.7±1.0 °C and 39.3±1.0 °C, respectively. These results show that binding occurs at a temperature T_0 that lies very close to the protein unfolding midpoint temperatures T_m of 43.3 and 40.5 °C, respectively.

Binding of Hsc70 to PGK in-cell is ATP-dependent Unlike non-specific sticking, productive Hsc70-substrate binding requires ATPase activity. Work in the literature has shown that a mutation to lysine at position 71 in the N-terminal nucleotide binding domain abrogates all ATPase activity.[22] Our hypothesis is that if the binding in figure 3 is ATP-dependent specific chaperone-substrate binding, then it should not be observed for the mutant mHsc70K71M. In order to check the specificity of binding in-cell, we constructed the ATPase-deficient mHsc70 mutant mHsc70K71M and showed that ATPase activity is abolished as compared to mHsc70 (SI Figure S6).

To conduct this control experiment, we transfected cells with ePGK and mHsc70K71M. Results showed that all four ePGK mutants showed only a small change in E_{FRET}. This change was only about ~4% with mHsc70K71M as compared to ~10% for mHsc70 binding to ePGK1-3. When compared to a control experiment with mEGFP and mCherry in-cell, a small E_{FRET} change of ~4% was also observed (SI Figure S7). Hence, the small 4% change observed with mHsc70K71M could be due to the interaction of mEGFP and mCherry. Moreover, no correlation between T_m and T_0 is observed for mHsc70K71M as was observed for mHsc70. These results show that binding in-cell of mHsc70 is indeed specific and ATP-dependent, unlike what is observed for the mHsc70K71M mutant.

Discussion

The few studies that have found evidence for co-localization of Hsp70 and Hsc70 during heat stress have hypothesized that both Hsc70 and Hsp70 could be responsible for stress response.[12,13] Our results show that mHsc70 indeed binds PGK during heat stress in a cooperative and ATP-dependent manner, as opposed to just sticking or aggregating at high temperature. We observed the onset of binding for the most stable PGK0 and 1 mutant, and fully-resolved binding curves for ePGK2 and 3.

Our previous characterization of the heat shock response of heat-inducible Hsp70 showed that Hsp70 binds PGK preemptively, with T_0 lower than T_m by as much as 4 °C.[8] In the case of Hsp70,

we hypothesized that Hsp70 is able to recognize and bind hydrophobic patches exposed during thermal fluctuations of the native state before full unfolding occurs.

Interestingly, for the case of constitutively expressed Hsc70 we observed that $T_0 \approx T_m$. The T_m for ePGK2 is 43.3±1.0 °C and the binding T_0 was 42.6±1.0 °C. Similarly, for ePGK3 the T_m was 40.5±1.0 °C and the binding T_0 was fit to 39.3±1.0 °C. Therefore, binding of substrate to Hsc70 is not preemptive in nature. Hsc70 is simply a holdase for PGK when it unfolds.

These contrasting behaviors of Hsp70 and Hsc70 can be rationalized by protein evolution. Hsc70 is present constitutively in the cell at concentrations as high as 1.5% of the total protein mass and among the top 25 proteins by copy number in U2-OS cells.[23,24] This translates to ~6 μM of protein in the cell according to the pax database (https://pax-db.org/). Even at physiological temperature (37 °C), native states of Hsc70 substrates will fluctuate to expose hydrophobic patches. If Hsc70 were to bind all such proteins, this could reduce the function of healthy proteins and more importantly, could remove valuable Hsc70 from the chaperone pool involved in cell homeostasis during normal cell function.[25] However, if binding occurs only upon complete substrate unfolding, such disadvantageous Hsc70 binding is minimized in the absence of heat shock, but Hsc70 can assist the heat-upregulated Hsp70 in the presence of heat shock.

Unlike Hsc70, Hsp70 is present in only very small amounts in a normal healthy cell at physiological temperature. Preemptive binding to transiently exposed hydrophobic stretches would not be a significant issue at such low concentrations. During heat stress, Hsp70 is upregulated in the cell to bind and rescue substrate proteins from unfolding and aggregation. The preemptive holdase mechanism of binding that was observed for heat-inducible Hsp70 has two additional benefits: 1) At elevated temperatures, proteins unfold more rapidly. Under such circumstances preemptive binding could inhibit the accumulation of unfolded substrate proteins. 2) Increased thermal fluctuations at elevated temperatures increase the probability of exposure of hydrophobic patches required for binding. Therefore, the preemptive holdase mechanism would be most effective at elevated temperatures, such as heat stress where the probability of such exposure is increased.

We hypothesize that heat-inducible Hsp70 evolved to bind native proteins via the preemptive holdase mechanism mainly during heat stress, whereas constitutively expressed Hsc70 binds substrates only upon complete unfolding under physiological conditions. Furthermore, we propose a mechanism where, as the cell is heated, Hsc70 is the first responder and binds proteins that are the first to unfold in the cell (Figure 5). At the onset of stress, the concentration of Hsp70 is low. During this time stress response by Hsc70 is potentially highly important for survival while the cell machinery upregulates Hsp70. Indeed, it has been shown that Hsc70 is required for the activation of

heat shock factor I (HSF1) which in turn switches on the Hsp70 upregulation machinery.[26] As more Hsp70 accumulates in the cell within 10s of minutes, the binding equilibrium shifts, and more substrates are bound by Hsp70 before they even unfold.

Some chaperones like GroEL are unfoldases.[27,28] Because unfoldases destabilize substrates and we have previously observed a stabilization of PGK in the cell,[29] this mechanism is not considered as a principal mechanism for Hsp70 or Hsc70 here.

Associated Content

Supporting Information

A PDF file containing Supplementary methods, Supplementary references, Supplementary Tables S1-S2, and Supplementary Figures S1-S6 is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.XXXXXX.

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Figure Captions

Figure 1: Plot comparing the distribution of donor/acceptor intensity below the onset of the PGK unfolding transition for the heat-inducible Hsp70 (Blue) and the constitutive Hsc70 (Orange). Points show experimental data. Solid marker and error bars show mean and standard deviation of the distribution. D/A, which could increase as a result of D binding to an unlabeled non-A protein, was only slightly higher for Hsc70 than for Hsp70.

Figure 2: Hsc70 is more stable in presence of ATP (light blue, squares) than without ATP (dark blue, circles) (A) wt-Hsc70 solvent exposure monitored by tryptophan fluorescence wavelength shift towards longer wavelength. The subsequent decrease of Trp fluorescence intensity above 45 °C is likely due to aggregation and partial re-burial of tryptophan (dotted line). (B) mHsc70 unfolding monitored by FRET resulting from aggregation of donor- and acceptor-labeled Hsc70. A similar stability increase in presence of ATP is observed. Filled markers connected by dashed lines show experimental data, and solid lines show fits to experimental data using equations 1-4 in Methods. Errors shown reflect the 1 standard deviation precision of the fit. Systematic errors are not accounted for.

Figure 3: Binding of mHsc70 to ePGK0 (black, circles), ePGK1 (dark red, squares), ePGK2 (red, diamonds) and ePGK3 (orange, triangles) in a U2-OS cell tracks protein stability. Filled markers connected by dashed lines show experimental data and solid lines show fits to experimental data using equations 1-4 in Methods. Each trace is an average data from 10 to 20 cells. The error bars shown reflect 1 standard deviation of cell-to-cell variation of the signal. Systematic errors are not accounted for.

Figure 4: Binding of inactive mutant mHsc70K71M to ePGK0 (black, circles), ePGK1 (dark red, squares), ePGK2 (red, diamonds) and ePGK3 (orange, triangles) in a U2-OS cell lacks ATP-specificity. Filled markers connected by dashed lines show experimental data and solid lines show fits to experimental data. The small signal does not depend on PGK melting temperature, and is likely due to weak mCherry-GFP association, similar to the small signal seen for the high-melting PGK mutant in Figure 4. Each trace is an average of 10 to 20 cells. The error bars shown reflect 1 standard deviation of cell-to-cell variation of the signal. Systematic errors are not accounted for.

Figure 5: Schematic depicting heat shock response interplay of Hsc70 (purple) and Hsp70 (green) to a substrate (red) in the cell. Left: A normal cell shown with three times more Hsc70 than Hsp70. Middle: As the temperature rises during heat stress, proteins partly or wholly unfold, and Hsc70 binds them. During this time the cell upregulates Hsp70. At high temperature Hsp70 also binds

substrates that only transiently expose hydrophobic patches (folded or mostly folded states). Right: As Hsp70 accumulates, binding equilibrium shifts and more Hsp70 is bound to substrates.

Figure 1

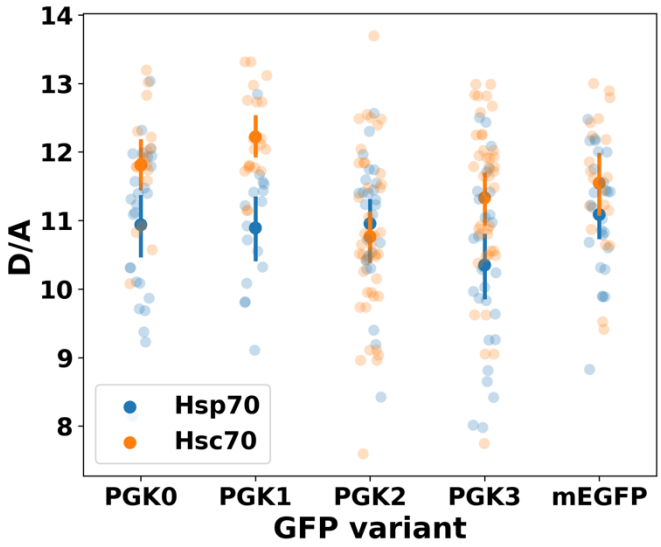
14
13
12
11
10
9
8
D/A
Hsp70
Hsc70
PGK0
PGK1
PGK2
PGK3
mEGFP
GFP variant

Figure 2

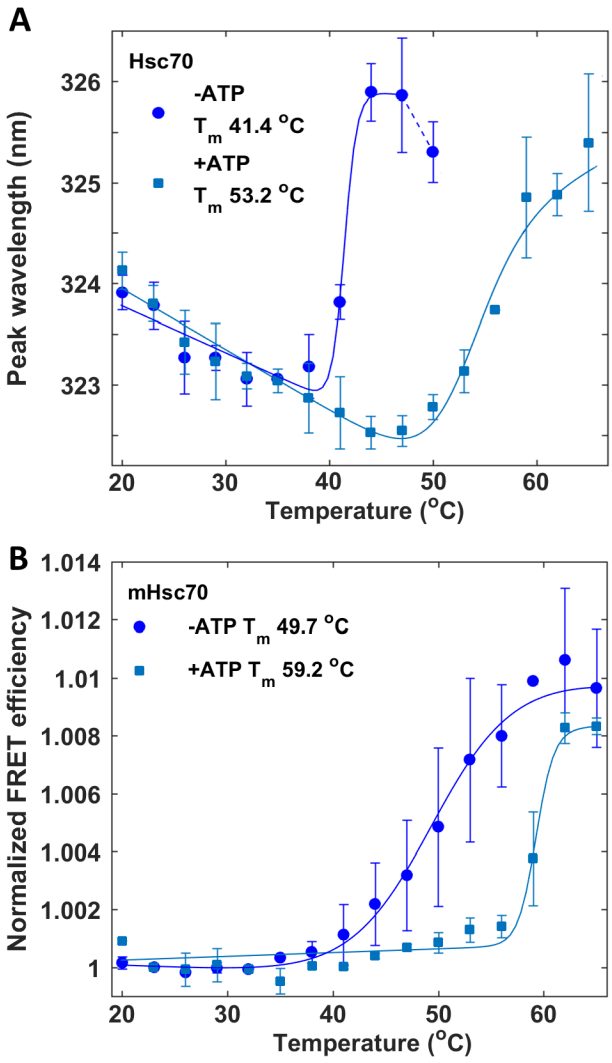
A
Hsc70
-ATP
T_m 41.4 °C
+ATP
T_m 53.2 °C
Peak wavelength (nm)
326
325
324
323
20
30
40
50
60
Temperature (°C)
B
mHsc70
-ATP T_m 49.7 °C
+ATP T_m 59.2 °C
Normalized FRET efficiency
1.014
1.012
1.01
1.008
1.006
1.004
1.002
1
20
30
40
50
60
Temperature (°C)

Figure 3

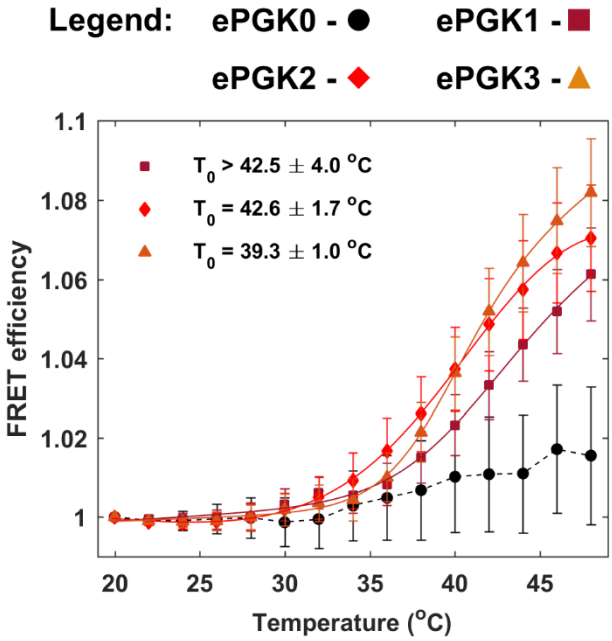
Legend: ePGK0 - ● ePGK1 - ■
ePGK2 - ◆ ePGK3 - ▲
T0 > 42.5 ± 4.0 °C
T0 = 42.6 ± 1.7 °C
T0 = 39.3 ± 1.0 °C
FRET efficiency
1.1
1.08
1.06
1.04
1.02
1
20
25
30
35
40
45
Temperature (°C)

Figure 4

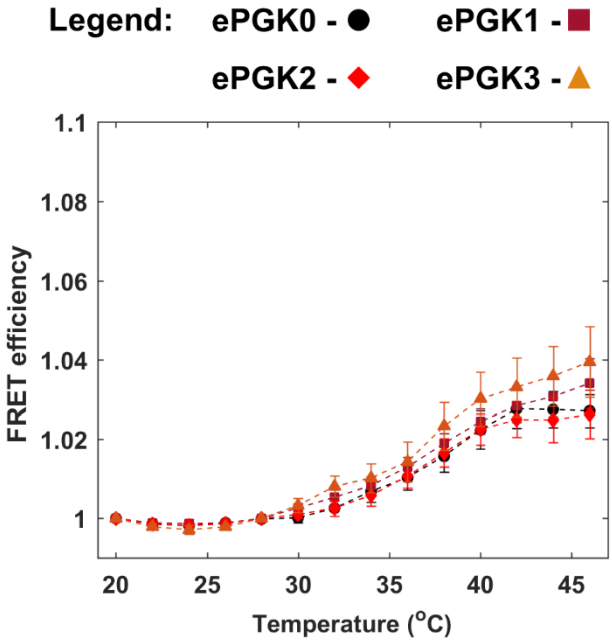
Legend:
ePGK0 -
ePGK1 -
ePGK2 -
ePGK3 -
FRET efficiency
1.1
1.08
1.06
1.04
1.02
1
20
25
30
35
40
45
Temperature (°C)

Figure 5