Hsp70 dynamics in vivo: effect of heat shock and protein aggregation

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Summary

The molecular chaperone Hsp70 interacts with misfolded proteins and also accumulates in the nucleus during heat shock. Using GFP-Hsp70 and fluorescence recovery after photobleaching, we show that Hsp70 accumulates in the nucleus during heat shock not only because its inflow rate increases but also because of a marked decrease in its outflow rate. Dynamic imaging also shows that GFP-Hsp70 has greatly reduced mobility when it interacts with organelles such as nucleoli in heat-shocked cells or the large inclusions formed from fragments of mutant huntingtin protein. In heat-shocked cells, nucleoplasmic Hsp70 has reduced mobility relative to the cytoplasm, whereas the ATPase-deficient mutant of Hsp70, Hsp70(K71E), is almost completely immobilized both in the nucleoplasm and the cytoplasm. Moreover, the Hsp70 mutant shows reduced mobility in the presence of diffusive huntingtin fragments with expanded polyglutamine repeats. This provides strong evidence that Hsp70 interacts not only with organelles but also with diffusive proteins in the nucleoplasm and cytoplasm during heat shock as well as with diffusive huntingtin fragments.

Key words: Hsp70, Mobility, Heat shock, Huntingtin

Introduction

The Hsc70s are among the most important families of molecular chaperones. Members of the Hsc70 family participate in numerous functions including folding of newly synthesized proteins, transport of proteins into mitochondria and the endoplasmic reticulum, formation of protein complexes in combination with the molecular chaperone Hsp90 and disaggregation of protein complexes such as clathrin-coated vesicles (Craig, 1989; Hartl et al., 1992). Hsp70 was initially discovered as one of the most prominent heat shock proteins exhibiting markedly increased expression when the cell is stressed by an increase in temperature or other stresses that lead to misfolding of proteins (Lindquist and Craig, 1988; Lindquist, 1986). An important function of Hsp70 is protecting cells from the deleterious effects of misfolded and aggregated proteins (Hartl et al., 1994) by preventing the misfolding of proteins required for critical cell activities, disaggregating misfolded proteins and presenting irreversibly damaged proteins to proteasomes for degradation.

One set of proteins targeted by Hsp70 during heat shock is present in the nucleoli of mammalian cells. Following heat shock, there is a marked increase in production of Hsp70 and much of this Hsp70 rapidly migrates to the nucleus of the cell and concentrates in the nucleoli (Welch and Feramisco, 1984; Pelham, 1984). Upon recovery from heat shock, the Hsp70 slowly returns to the cytosol. Although this phenomenon was described many years ago, its mechanism is only beginning to be deciphered. Mutations in two regions of Hsp70 reduce its ability to migrate to the nucleus (Knowlton, 1999; Knowlton, 2001), whereas phosphorylation of tyrosine 524 increases migration (Knowlton et al., 2000). However, little is known about the rates at which Hsp70 enters and leaves the nucleus during the various phases of heat shock. For example, it is not known whether Hsp70 in the nucleus of stressed or unstressed cells is in rapid equilibrium with Hsp70 in the cytosol; nor is it known whether the interaction of Hsp70 with diffuse proteins in the nucleus differs from its interaction with proteins that are concentrated in the nucleolus.

A similar question arises in regard to the interaction of Hsp70 with protein aggregates that form in a number of degenerative neurological disorders. A wide array of neurodegenerative diseases, including Alzheimer's disease, Parkinson disease, prion disease, amyotrophic lateral sclerosis, and Huntington's disease (HD) show a common feature – inclusion and deposition of abnormal protein in neurons of vulnerable brain regions (Taylor et al., 2002). In HD, these protein inclusions are caused by the accumulation of proteins or protein fragments that contain long stretches of glutamines that, in turn, result from a pathological expansion of the CAG repeat in the coding region of the huntingtin (*Htt*) gene. In HD and in other 'polyglutamine' neurodegenerative diseases, polyglutamine-containing fragments of disease-protein accumulate in inclusions found both in the nucleus and cytoplasm. These inclusions might be protective, isolating the aggregated proteins from the rest of the cell (Yang et al., 2002; Kawaguchi et al., 2003; Taylor et al., 2003a). However, these inclusions might cause harm by sequestering proteasomes,

Hsp70, transcription factors or proteins involved in endocytosis (Taylor et al., 2003b; Zhou et al., 2003).

When Htt fragments containing expanded glutamine repeats are expressed in tissue culture cells they form cytoplasmic inclusions that resemble aggresomes (Waelter et al., 2001). Production of aggresomes has been proposed to be a generalized response of cells to the formation of aggregated proteins (Johnston et al., 1998; Garcia-Mata et al., 1999). Aggresomes have a number of interesting properties: they are generated by transport of small aggregates of misfolded protein toward the microtubule organizing center (MTOC); in addition to the aggregated protein itself, they recruit a number of different molecular chaperones including Hsp70 and J-domain proteins (homologs of the DnaJ family of proteins) that present substrates to Hsp70; and they are associated with proteasomes suggesting that they may be centers where aggregated proteins are degraded (Garcia-Mata et al., 1999).

There is strong evidence that Hsp70 and J-domain proteins play an important role in protecting cells from the deleterious effects of the misfolded, aggregated proteins found in association with neurodegenerative diseases. For example, overexpression of Hsp70 suppresses degeneration and improves motor function in a transgenic mouse model of SCA1 (Cummings et al., 2001). Similarly, overexpression of Hsp70 strongly suppresses the toxicity of expanded polyglutamine and mutant α-synuclein in *Drosophila melanogaster* models of SCA3 and Parkinson's disease, respectively (Warrick et al., 1999; Auluck et al., 2002). Hsp70 associates with aggregated proteins in these diseases in what has been interpreted as an effort to rid the cell of the offending protein (Muchowski, 2002). However, it is not clear whether the protective effect of Hsp70 is because of direct interaction with misfolded protein or, alternatively, a result of Hsp70's inhibitory activity on apoptosis (Taylor et al., 2003a; Schaffar et al., 2004).

A recent study reported that the mobility of green fluorescent protein (GFP)-Hsp70 interacting with polylutamine inclusions or nucleoli (during heat shock) was the same as that of GFP-Hsp70 in the cytosol of untreated cells (Kim et al., 2002). Moreover, this mobility was only about one-fourth of that found using truncated Hsp70 lacking a substrate binding site, which, presumably, diffuses freely in the cytosol. This is somewhat surprising because the mobility of freely diffusing proteins is typically orders of magnitude faster than the mobility of proteins bound to cellular organelles; in the latter case the mobility would presumably be a measure of the rate of dissociation of the protein form the organelle (Coscoy et al., 2002). Because Hsp70 associated with the inclusions showed about the same mobility as Hsp70 interacting with unfolded proteins in the cytosol, Kim et al. suggested that Htt inclusions are dynamic rather than static structures, an unexpected finding because the Htt in these inclusions, like ataxin3 in its inclusions, is completely immobilized (Kim et al., 2002; Chai et al., 2002). However, ataxin 1 inclusions show both fast and slow exchanging components (Stenoien et al., 2002).

In the present study, we investigated a number of outstanding questions involving Hsp70 using fluorescence recovery after photobleaching (FRAP) with GFP-Hsp70 and GFP-Hsp70(K71E), an Hsp70 mutant unable to hydrolyze ATP (O'Brien et al., 1996). Our results showed, first, that accumulation of GFP-Hsp70 in the nucleus during heat shock is caused not only by an increase in the rate of Hsp70 transport into the nucleus but also by a significant decrease in its rate of transport out of the nucleus. Second, in contrast to the results of Kim et al. (Kim et al., 2002), GFP-Hsp70 does not always show the same mobility nor does it always show the same interaction with organelles. The mobility of GFP-Hsp70 bound to either nucleoli during heat shock or polyglutamine inclusions is much less than the mobility of GFP-Hsp70 in the cytosol of control cells, and whereas GFP-Hsp70 is localized throughout the nucleoli, it only binds to the surface of the inclusions. Finally, we found that Hsp70 not only interacts with nucleoli during heat shock but also with diffuse cytoplasmic and nucleoplasmic proteins. Similarly it not only interacts with polyglutamine inclusions, but also with diffuse polyglutamine fragments in the nucleus. This latter interaction might play a key role in the ability of Hsp70 to protect cells from the effects of heat shock and aggregated Htt fragments.

Materials and Methods

Plasmids

Hsp70 and Hsp70(K71E) (Rajapandi et al., 1998) were sublconed from pET21a into pEGFP-C3 (Clontech, CA) or into pFlag-CMV-2 (Sigma) using *Hind*III and *Bam*HI sites. The Htt polyglutamine constructs containing glutamine repeat expansions with 25 (Q25) or 72 glutamines (Q72) had either an enhanced GFP (EGFP)-tag or a myc-tag on the C-terminal end. The Htt constructs were sublconed into the N1-RFP vector, obtained as a gift from R. Tsien (Stanford University, Palo Alto, CA) (Campbell et al., 2002). The EGFP-Htt constructs and the EGFP-tagged cAMP response binding protein (CBP) were gifts from A. Tobin (UCLA) and H. Paulson (University of Iowa), respectively.

Tissue culture and immunostaining

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biofluids, MD) supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 unit/ml) and streptomycin (100 unit/ml) in a humidified incubator with 5% CO_2 at 37°C. Cells were heat shocked in a humidified incubator with 5% CO_2 at 43°C for 1 hour and either imaged immediately after removal from the incubator or returned to the 37°C incubator to follow recovery from heat shock. Cells were treated with 1 mM or 5 mM dithiothreitol (DTT) following heat shock for 15 minutes at 37°C (Nehls et al., 2000). Cells were transfected with the plasmid DNA using Fugene6 (Roche Diagnostics, IN). Cotransfections using a GFP-vector and either a myc-tagged or flagged-tagged vector were always done using half as much of the GFP-vector as the non-fluorescent vector. Apoptosis was measured 72 hours after transfection by fixing and staining the cells with cleaved caspase 3 antibody (Cell Signaling Technology, MA).

Confocal microscopy

Cells grown on two-chamber 25-mm^2 cover slips (Labtek, NY) were imaged on a Zeiss LSM 510 confocal microscope. GFP-clathrin was imaged and photobleached using 488-nm laser light with a 40×, 1.4 NA objective. RFP-Htt was imaged using a 543-nm laser light. A defined region was photobleached at high laser power resulting in 50-80% reduction in the fluorescence intensity. The fluorescence recovery was monitored by scanning at low laser power. To get time points in the millisecond time range when measuring the fluorescence recovery of photobleached, highly diffusable protein, the fluorescence scanning was modified in two major ways which decreased the scan time. First, the pixel density was decreased from 512×512 to 128×128. Second, the scanning distance along the ordinate axis was

markedly reduced. When data sets were compared, identical conditions were used in photobleaching the cells including the number of bleaches, the area of the photobleach region and the time course of imaging at low laser power.

Data analysis

For each experimental condition, a minimum of ten data sets were averaged to get the mean and standard deviation for each time point. The fluorescence-intensity data in each experiment were normalized by setting the maximum fluorescence to 100% and the minimum to 0%. The very low laser intensity used in scanning the cell after the initial photobleach did not cause significant bleaching during our experiments and therefore no correction was necessary for this effect. Even though the fluorescence of the total GFP pool in the cell was unaffected by scanning, the total recovery in most experiments was only about 80% of the initial fluorescence because about 20% of the total GFP pool in the cell was bleached by the initial bleach.

Results

Characterization of GFP-Hsp70

To study the properties of Hsp70, we constructed an N-terminal GFP-Hsp70 and then established whether it still maintains normal Hsp70 activity. One of the activities of Hsp70 as a molecular chaperone is to protect the cell against the toxic effects of Htt-aggregation (Merienne et al., 2003). Therefore, we examined whether GFP-Hsp70 is able to protect the cell against the apoptotic effects of polyglutamine aggregation. We found that GFP-Hsp70 protected cells to a similar extent as flagged-tagged Hsp70 from apoptosis induced by expression of Htt(Q72) (Fig. 1A). As expected, expression of Htt(Q25) did not cause significant apoptosis of transfected cells. This clearly demonstrates that GFP-Hsp70 is physiologically active.

Another way of validating that GFP-Hsp70 retains the activity of unmodified Hsp70 is to show that it migrates to the nucleus and, in particular, to nucleoli following heat shock. Fig. 1B shows the distribution of GFP-Hsp70 expressed in HeLa cells before and after heat shock. Before heat shock about 60% of the GFP-Hsp70 was present in the cytosol, although some was also present in the nucleus, particularly in cells showing a high overall expression of GFP-Hsp70. However, even in these cells, the GFP-Hsp70 was completely excluded from the nucleoli before heat shock. By contrast, immediately following heat shock, there was a marked increase in the level of GFP-Hsp70 present in the nucleus and furthermore, much of this GFP-Hsp70 became concentrated in the nucleoli.

It is not clear whether the changes in nuclear concentrations of Hsp70 during heat shock are only owing to changes in the inflow rate of Hsp70 or whether both inflow and outflow rates change during heat shock and recovery. Because GFP-Hsp70 can be photobleached, we addressed this question by measuring the rates of Hsp70 entry into and exit from the nucleus under different conditions. Completely bleaching the total volume of either the cytosol or the nucleus (see Fig. 2 legend) allowed us to determine the percent of Hsp70 that flowed into or out of the nucleus over a given period of time. Before heat shock, Hsp70 clearly passed across the nuclear membrane in both directions, its rate of transport was relatively slow (Fig. 2A-C). Quantification of these data by averaging over a large number of cells showed that in 15 minutes about 6% of the Hsp70 in the cytosol passed into the nucleus and

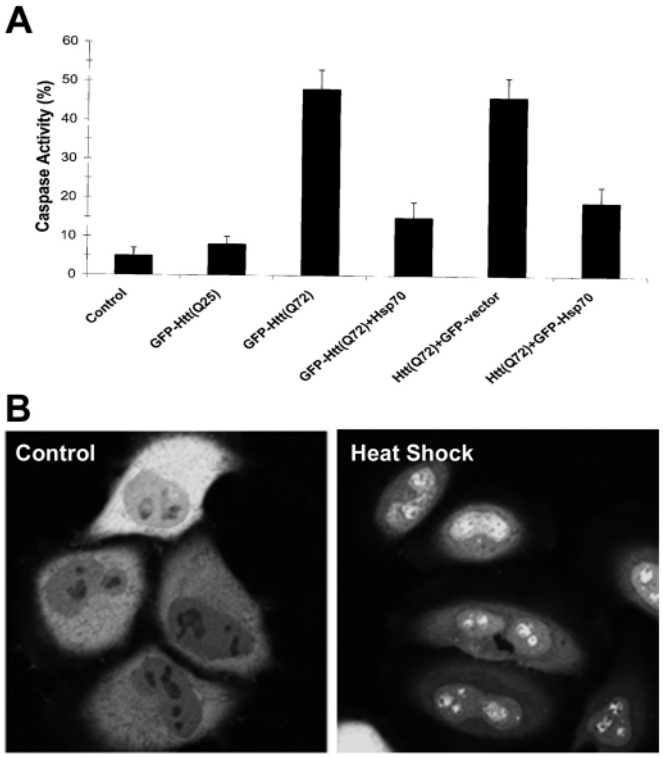

Fig. 1. Properties of GFP-Hsp70. (A) Protection of GFP-Hsp70 against apoptosis from the expression of Htt(Q72). The extent of apoptosis was measured 72 hours after transfection in control cells and cells expressing GFP-Htt(Q25), GFP-Htt(Q72), GFP-Htt(Q72) and flag-tagged Hsp70, myc-tagged Htt(Q72) and GFP vector, and myc-tagged Htt(Q72) and GFP-Hsp70. Apoptosis was measured by immunostaining for cleaved caspase 3 using rhodamine-conjugated secondary antibody. The data represent an average of three experiments in which 100 GFP cells were analyzed for positive caspase staining. (B) Distribution of GFP-Hsp70 in control (left) and heat-shocked cells (right).

about 40% of the Hsp70 in the nucleus passed into the cytosol (Fig. 3). Given that the cytosol contains about 60% of the total Hsp70 in the cell and assuming that the nucleus is about one-sixth of the total cell volume, this means that roughly equal amounts of Hsp70 are passing in both directions across the nuclear membrane, as expected under steady-state conditions.

Fig. 2D-F shows a similar experiment carried out immediately after heat shock. Quantification of these data in Fig. 3 shows that following heat shock the inflow rate of Hsp70 into the nucleus doubled, while the outflow rate was reduced by about two-thirds. Thus, the rapid net accumulation of Hsp70 in the nucleus following heat shock results not only from increased inflow, but also from markedly reduced outflow. This results in the nucleus being much brighter than the cytosol (Fig. 4A). Six hours following heat shock, during the recovery phase, the outflow rate increased beyond the basal level in untreated cells while the inflow rate decreased markedly. By contrast, the transport of GFP across the nuclear membrane, which is very rapid, was unaffected by heat shock (data not shown). It should be noticed that comparison of inflow rates and outflow rates necessitates a correction for the relative volume of the nucleus and cytosol. However, comparison of the relative values of inflow and outflow rates requires only that

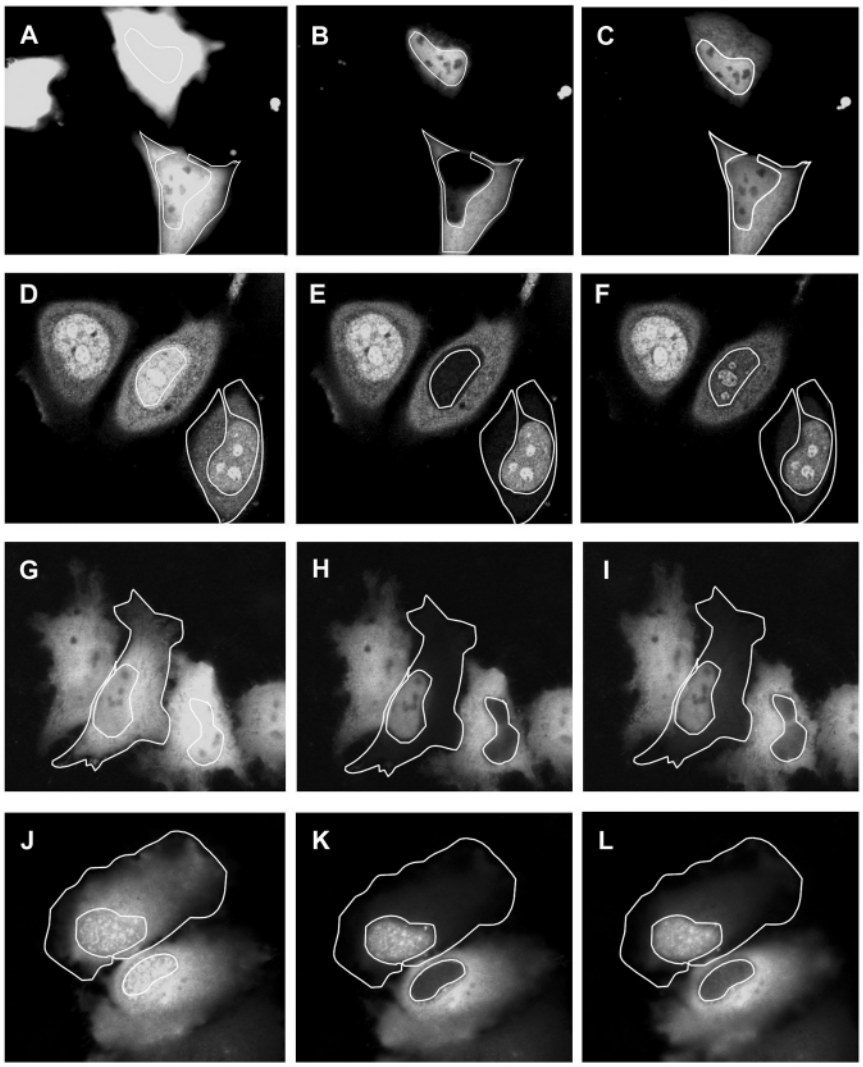

Fig. 2. The transport of (A-F) GFP-Hsp70 and (G-L) GFP-Hsp70(K71E) into and out of the nucleus in control (A-C,G-I) and heat-shocked cells (D-F,J-L). Cells before (A.D,G,J) photobleaching, immediately after (B,E,H,K) photobleaching and 15 minutes following (C,F,I,L) photobleaching. Outlined areas indicate photobleached regions. Fluorescence of pre-bleached cells was intensified during imaging to insure that the fluorescence signal of the post-photobleached cells was sufficiently high to accurately measure transport.

the relative volumes of the nucleus and cytosol remain constant following heat shock. Therefore, these data show that the level of Hsp70 in the nucleus, under various conditions, is controlled by changes in both inflow and outflow rates of Hsp70 from the nucleus.

In contrast to the results obtained with GFP-Hsp70, the migration of the ATPase-inactive mutant of Hsp70, GFP-Hsp70(K71E), was much less affected by heat shock. Before heat shock, the distribution of GFP-Hsp70(K71E) was very similar to GFP-Hsp70 (Fig. 2G-I). However, after heat shock there was no significant accumulation of GFP-Hsp70(K71E) in the nucleus and nucleolus (Fig. 2J-L), indicating that ATP binding or hydrolysis must play an important role in Hsp70 migration. Quantification of the in- and out-rates shows that GFP-Hsp70(K71E) entered into and exited from the nucleus much more slowly than GFP-Hsp70 in non heat-shocked cells (Fig. 3). Furthermore, following heat shock, the rate of GFP-Hsp70(K71E) entry into and exit from the nucleus became unmeasurably slow, so that there was almost no change in the distribution of GFP-Hsp70(K71E) between the nucleus and the cytosol following heat shock (Fig. 4B). Thus, particularly in heat shock, the ability of Hsp70 to rapidly flow into and out of the nucleus depends on an intact ATP-hydrolysis-site.

Furthermore, we found that expression of flag-tagged Hsp70(K71E) in control cells markedly decreased the rates at which GFP-Hsp70(K71E) itself entered and exited from the nucleus (data not shown). This dominant influence suggests that Hsp70(K71E) might form a complex with GFP-Hsp70(K71E) and prevent it from being transported into and out of the nucleus in a normal manner.

Mobility of Hsp70 during heat shock

We next investigated the local mobility of GFP-Hsp70(K71E) in control and heat-shocked cells by determining its rate of recovery-of-fluorescence following photobleaching. We first determined the mobility of GFP alone, which is considered completely mobile. We obtained a half-life of recovery of 0.3 seconds for fluorescence recovery of GFP in the cytosol (Fig. 5A), but because photobleaching of the freely diffusable material did not just occur in the volume that we bleached, the fluorescence recovery is not just a single exponential (Coscoy et al., 2002). Therefore, the measured half-life does not provide an accurate diffusion constant. Instead, to compare different recovery rates, we used the measured half-lives of fluorescence recovery as a relative measure, comparing all of the other half-lives for fluorescence recovery to the half-life for GFP alone, using identical settings when performing the photobleaches. We first measured the mobilities of Hsp70 in the cytoplasm and nucleus and found that in all cases the half-life of fluorescence recovery was less than 1 second. The mobilities of GFP-Hsp70 in both the cytoplasm (Fig. 5A) and nucleoplasm (Fig. 5B) were less than a factor of 2 higher than the mobility of GFP. This slightly higher value can in part be attributed to the three-fold higher molecular weight of GFP-Hsp70 compared to GFP because diffusion is roughly proportional to the cube root of the molecular weight (Reits and Neefjes, 2001). In the nucleus, the plateau level of GFP-fluorescence-recovery after photobleaching is slightly lower than the plateau level of GFP-Hsp70-fluorescence-recovery because, as described in Material and Methods, as we bleach, fluorescent material outside of the bleach flows into the bleached area, and because GFP has a higher mobility than GFP-Hsp70, this effect occurs to a greater extent with GFP than with GFP-Hsp70.

We next investigated the mobility of GFP-Hsp70 after heat

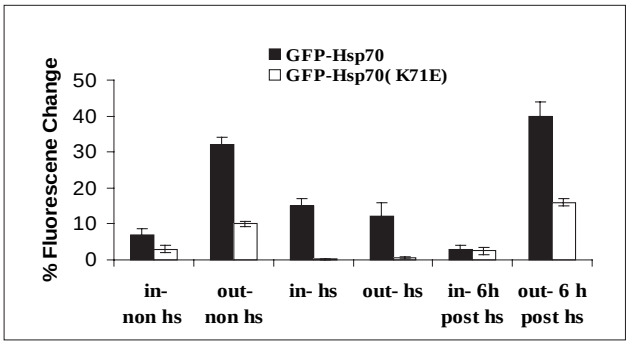

Fig. 3. The relative rate of transport into and out of the nucleus before, immediately following, and six-hours post heat shock of GFP-Hsp70 and GFP-Hsp70(K71E). Transport out of the nucleus was determined by bleaching the cytosol and then measuring the percent of the nuclear fluorescence that entered the bleached cytosol in 15 minutes. Transport into the nucleus was determined by bleaching the nucleus and then measuring the percent of the cytosolic fluorescence that entered the bleached nucleus in 15 minutes. Either the nucleus or the cytoplasm was bleached multiple times until the GFP fluorescence was essentially unmeasurable. The cells were imaged with the pinhole set to 12 μm to visualize the entire depth of the cell. To increase the resolution when the in and out-rates of GFP-Hsp70 were measured, images were scanned every 15 seconds using a pixel depth of 12×12 bits. To directly compare in and out-rates, data were corrected by assuming that one-sixth of the cell volume is occupied by the nucleus. Note that this correction is not necessary when comparing either in-rates with each other or out-rates with each other under varied conditions. Data from at least 10 cells were collected for each condition and their average and standard deviation were determined. GFP-Hsp70, black bars, and GFP-Hsp70(K71E), white bars.

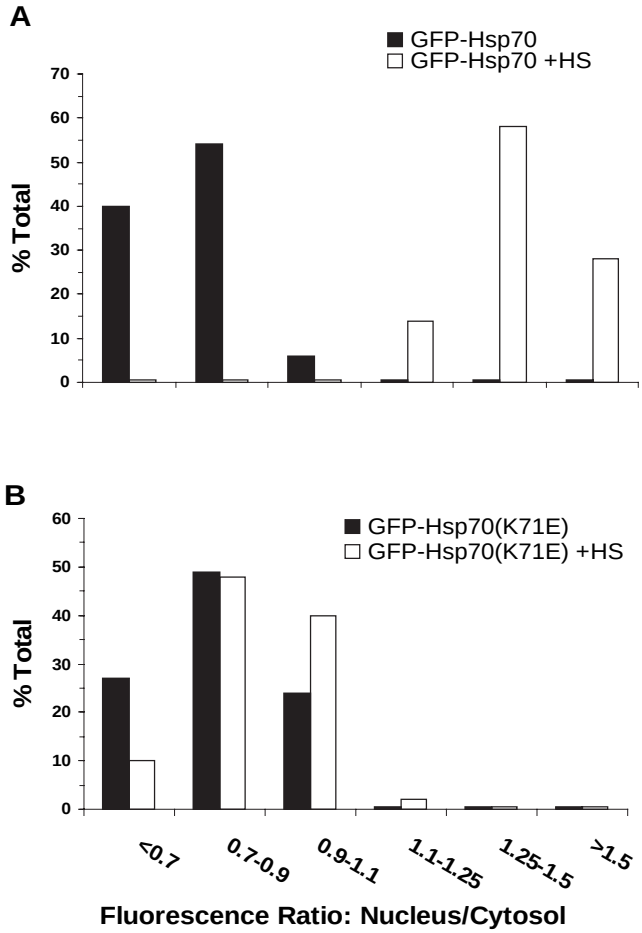

Fig. 4. Cellular distribution of GFP-Hsp70 and GFP-Hsp70(K71E) in control and heat-shocked cells. HeLa cells transfected with GFP-Hsp70 (Fig. 4A) or GFP-Hsp70(K71E) (Fig. 4B) were imaged before (black bars) and immediately following (white bars) heat shock. The average fluorescence intensities of the cytosol and the nucleus were measured and the ratio of the fluorescence of the nucleus relative to the cytosol was calculated. A minimum of 250 cells per experimental condition were imaged and the percent of cells with each Hsp70 distribution range was determined. Hsp70 distribution was calculated as percentage of the total cells imaged.

shock. We found no detectable change in the mobility of GFP-Hsp70 in the cytoplasm after heat shock, with the half-life of its fluorescence recovery still less that 1 second (Fig. 6). By contrast, following heat shock, the mobility of GFP-Hsp70 associated with nucleoli was decreased by more than one order of magnitude, with a half-life of fluorescence recovery of about 20 seconds (Fig. 6) and even the GFP-Hsp70 in the nucleoplasm showed a significant reduction in mobility with a half-life of fluorescence recovery of about 5 seconds (Fig. 6A). Therefore, the mobility of GFP-Hsp70 in the nucleoplasm is reduced by 80% following heat shock. By contrast, the mobility of GFP in the nucleoplasm was not altered after heat shock (Fig. 6), showing that there is not a general decrease in the mobility of proteins located in the nucleoplasm. Therefore, we have obtained strong evidence that Hsp70 interacts with diffuse nuclear proteins and also with proteins in the nucleoli during heat shock.

To obtain further evidence of this interaction, we measured the mobility of GFP-Hsp70(K71E) before and after heat shock, reasoning that, because it was, in effect, in a nucleotide-free state (Rajapandi et al., 1998), it should bind substrates more stably than Hsp70. In control cells, the mobility of GFP-Hsp70(K71E) was only slightly less than that of GFP-Hsp70 both in the cytosol and the nucleus (Fig. 5). This slight decrease in mobility might be owing to oligomerization of the GFP-Hsp70(K71E) in the cytosol (Newmyer et al., 2001). In contrast to its high mobility in untreated cells, following heat shock, there was a remarkable decrease in the mobility of GFP-Hsp70(K71E). As shown in Fig. 6B, following photobleaching, the fluorescence of GFP-Hsp70(K71E) in the nucleoplasm showed almost no recovery. Even 30 minutes after photobleaching, the bleached region was still very apparent. There was also a large immobilized fraction of GFP-Hsp70(K71E) in the cytosol following heat shock, whereas the remaining fraction recovered on a relatively slow time scale (Fig. 6B). These effects were completely reversible after the cells recovered from heat shock. The presence of flag-tagged Hsp70(K71E) also profoundly decreased the mobility of GFP-Hsp70 both in the cytoplasm and nucleoplasm of heat shocked cells, but had no effect on the mobility of GFP alone (data not shown). Taken together, these data strongly suggest that Hsp70 interacts with diffuse nuclear and cytosolic proteins during heat shock and also with proteins in the nucleoli.

Mobility of Hsp70 in the presence of Htt fragments

We next investigated the mobility of GFP-Hsp70 in cells

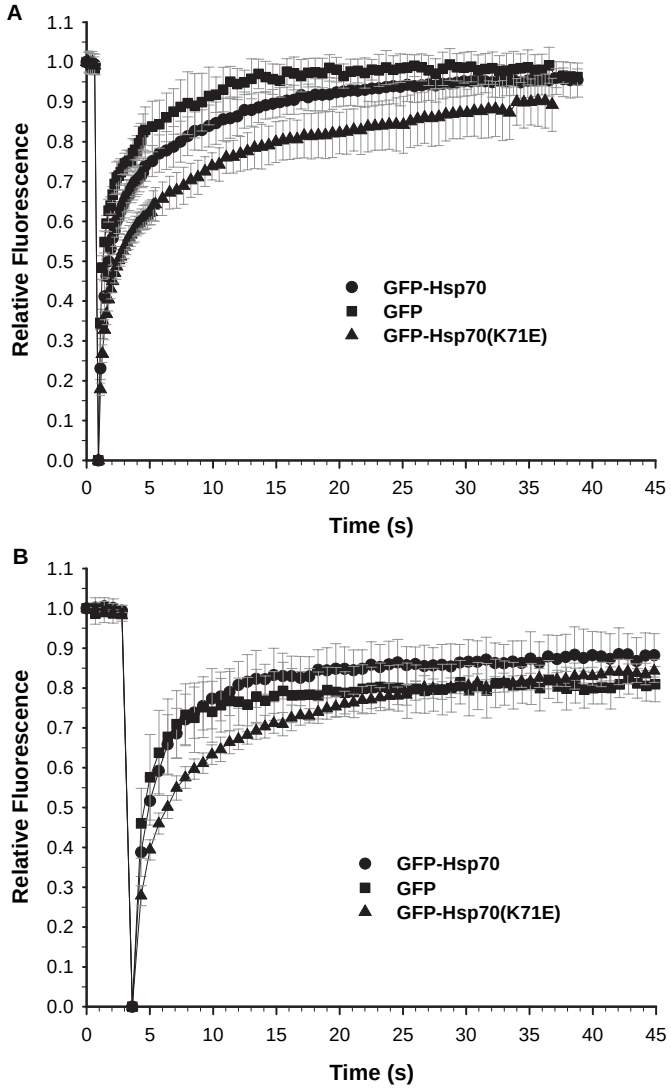

Fig. 5. Mobility of GFP-Hsp70 and GFP-Hsp70 (K71E) relative to GFP in (A) cytosol and (B) nucleus. GFP or GFP-Hsp70 were bleached and the fluorescence recovery after photobleaching was measured. The fluorescence recovery after photobleaching is shown for GFP (■), GFP-Hsp70 (●) and GFP-Hsp70(K71E) (▲).

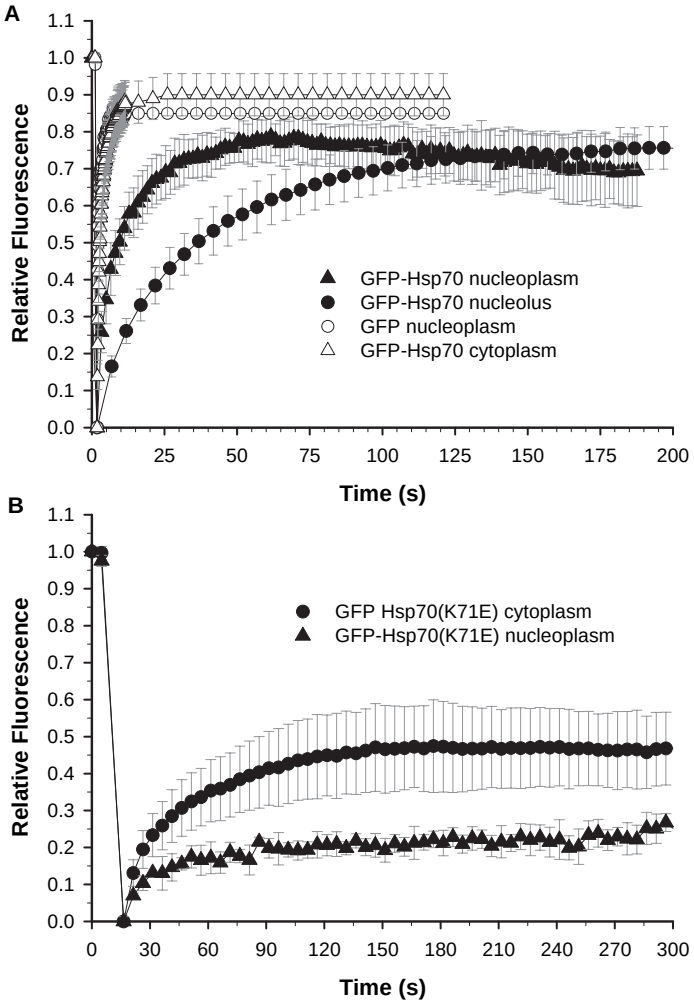

Fig. 6. Heat shock affects mobility of GFP-Hsp70 in the nucleus and GFP-Hsp70(K71E) in the nucleus and cytoplasm. HeLa cells were heat shocked and the fluorescence recovery after photobleaching was measured. (A) fluorescence recovery of GFP vector in the nucleus(○), GFP-Hsp70 in the cytoplasm (△), GFP-Hsp70 in the nucleoplasm (▲) and GFP-Hsp70 in the nucleolus (solid circles). (B) fluorescence recovery of GFP-Hsp70(K71E) in the cytoplasm (●) and nucleoplasm (▲).

expressing fragments of Htt protein that contains pathological polyglutamine expansions. HeLa cells transiently transfected with GFP-Htt(Q72) show small, bright green inclusions formed in a small percentage of the cells 24 hours after transfection and in about 15% of the cells 48 hours after transfection. By 48 hours, the small aggregates clustered together to form a large cytoplasmic perinuclear inclusion in many of the cells, while a minority had Htt(Q72) inclusions in the nucleus. When we photobleached GFP-Htt(Q72) inclusions in either the nucleus or the cytoplasm there was no recovery of fluorescence. Similarly, in an experiment in which we measured the fluorescence loss in photobleaching (FLIP) by repetitive bleaching of GFP-Htt(Q72) in the cytosol or nucleoplasm, there was no decrease in fluorescence in the inclusions (data not shown). Therefore, as previously reported (Kim et al., 2002), GFP-Htt in the inclusions is immobilized and is not in equilibrium with the free GFP-Htt in the cytosol or nucleoplasm.

We then investigated the mobility of GFP-Hsp70 associated with polyglutamine inclusions. In these experiments, we used RFP-Htt(Q72) to enable simultaneous observation of the inclusions and the GFP-Hsp70. We found that GFP-Hsp70 formed shells around the large juxtanuclear inclusions at the MTOC (Fig. 7A-C). The observation that Hsp70 forms shells around Htt inclusions is in agreement with earlier observations showing that Hsp70 forms shells around aggresomes rather than completely penetrating them (Garcia-Mata et al., 1999). We also observed that similar shells of GFP-Hsp70 formed around the RFP-Htt(Q72) in nuclear inclusions (Fig. 7D-F). To ensure that the inclusions were actually in the nucleus, the nucleus was stained with 4′,6′-diamidino-2-phenylindole (DAPI) and the inclusions were visualized in three dimensions.

The GFP-Hsp70 in the shells was photobleached, resulting

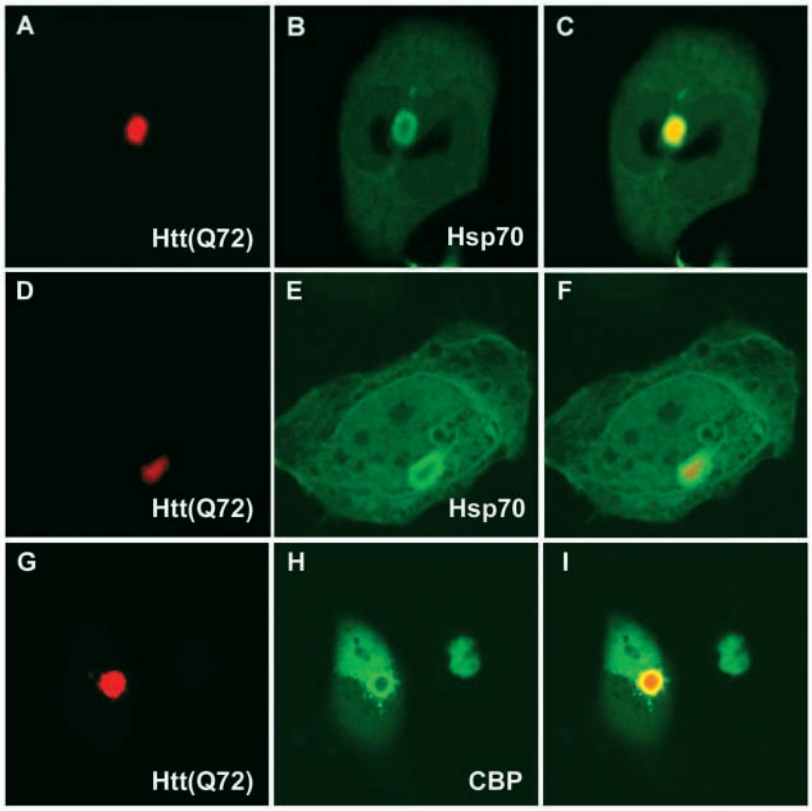

Fig. 7. GFP-Hsp70 or GFP-CBP shells around RFP-Htt(Q72) inclusions. HeLa cells cotransfected with RFP-Htt(Q72) and either GFP-Hsp70 or GFP-CBP were imaged 48 hours after transfection. (A,D,G) RFP-Htt(Q72); (B) GFP-Hsp70; (H) E:GFP-CBP. Merged images are C, F and I.

in about two-thirds of the GFP-Hsp70 around the inclusions recovering with a half-life of about 30 seconds (Fig. 8), even slower than the rate of recovery we observed for GFP-Hsp70 associated with nucleoli. Whereas one-third of the GFP-Hsp70 associated with inclusions appeared immobile on short time scales, FLIP experiments, in which GFP-Hsp70 in the cytosol was continually bleached, showed that even this GFP-Hsp70 was in slow equilibrium with cytosolic GFP-Hsp70 (data not shown).

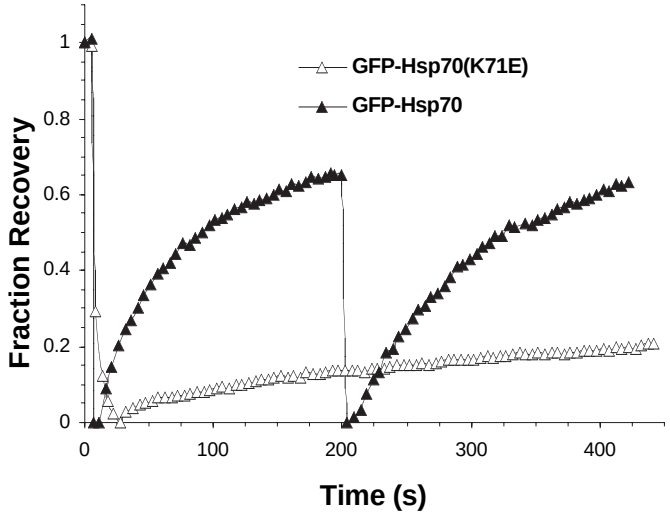

Fig. 8. Interaction of GFP-Hsp70 and GFP-Hsp70(K71E) with Htt inclusions. Cells were transfected with RFP-Htt(Q72) and either GFP-Hsp70 or GFP-Hsp70(K71E). GFP-Hsp70 (▲) or GFP-Hsp70(K71E) (△) shells around Htt inclusions were photobleached.

It has been reported that the transcription factor CBP is sequestered by polyglutamine inclusions (Steffan et al., 2000; McCampbell et al., 2000), leading to the suggestion that functional depletion of CBP by insoluble Htt might contribute to the pathogenesis of HD and related diseases (McCampbell and Fischbeck, 2001). We found that GFP-CBP, like GFP-Hsp70, formed shells around the RFP-Htt(Q72) inclusions both in the cytoplasm (Fig. 7G-I) and in the nucleus (data not shown). Both FRAP and FLIP experiments showed that the GFP-CBP was completely immobile (data not shown), in agreement with the results of Chai et al. (Chai et al., (2002). Therefore, both GFP-Hsp70 and GFP-CBP form shells around the inclusions but, whereas two-thirds of the Hsp70 is able to detach from the inclusions with a half-life of about 30 seconds, the CBP is completely immobilized. Similar to CBP, we found that the occasional shells formed by GFP-Hsp70(K71E) around the Htt inclusions were completely immobile (Fig. 8). This indicates that ATP-binding and hydrolysis by Hsp70 is important for dissociation of Hsp70 from Htt inclusions.

We next tested whether the presence of Htt has any effect on the mobility of GFP-Hsp70 or GFP-Hsp70(K71E) in the cytosol or nucleoplasm. We found that the presence of Htt(Q72) had no effect on the mobility of GFP-Hsp70, neither in the nucleus nor the cytosol (Fig. 9). Furthermore, we observed no effect of Htt(Q72) on the mobility of GFP-Hsp70(K71E) in the cytosol. However, the presence of Htt(Q72) did affect the mobility of GFP-Hsp70(K71E) in the nucleoplasm. Fig. 9 shows that the half-life of fluorescence recovery was about 1.5 seconds in the absence of Htt(Q72), whereas in the presence of Htt(Q72) the half-life of recovery was about 3 seconds. This is a specific effect of Htt(Q72); it does not occur with Htt(Q25), a Htt protein carrying a shorter wild-type stretch of polyglutamine (data not shown). Therefore, these data show that Hsp70 interacts either directly or indirectly with diffuse aggregates of Htt fragments and also with Htt fragments in inclusions.

Discussion

In this study we used GFP-Hsp70 to directly investigate various aspects of Hsp70 activity in untreated cells, heat-shocked cells and cells expressing fragments of polyglutamine expanded Htt fragments. Numerous immunostaining studies have shown that Hsp70 accumulates in the nucleus during heat shock. However, these studies followed only net movement of Hsp70. By using GFP-Hsp70 and FRAP, we were able to measure the rates of Hsp70 movement into and out of the nucleus both before and after heat shock. Our results show that, in untreated cells, Hsp70 in the nucleus exchanges with

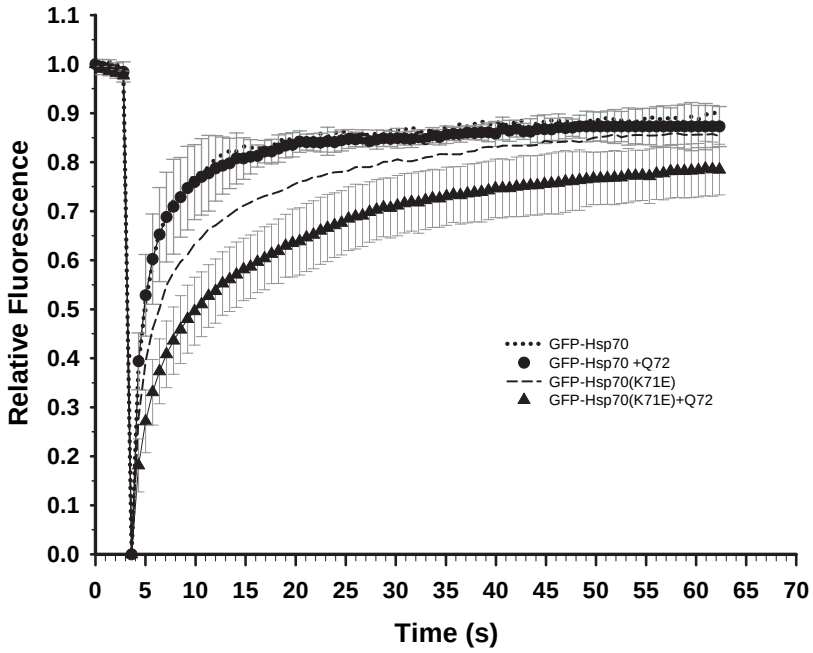

Fig. 9. Effect of Htt(Q72) on the mobility of GFP-Hsp70 and GFP-Hsp70(K71E) in the nucleoplasm. HeLa cells were cotransfected with myc-tagged Htt(Q72) and either GFP-Hsp70 (●) or GFP-Hsp70(K71E) (▲) and then imaged 48 hours after transfection. The dotted line, superimposed on the solid circles, was obtained for the GFP-Hsp70 data in Fig. 5B while the dashed line was obtained for the GFP-Hsp70(K71E) data in Fig. 5B. The data sets in Fig. 5B were obtained under the identical conditions as the data obtained in the presence of Htt.

cytosolic Hsp70 with a half-life of about 30 minutes. Following heat shock, there is a two-fold increase in the rate of nuclear import of Hsp70 and a three-fold decrease in the rate of nuclear export. Furthermore, the migration of Hsp70 out of the nucleus during recovery from heat shock is caused not only by a decrease in the rate of inflow but also by a marked increase in the outflow rate to a level greater than that obtained in untreated cells. This is the first demonstration that the cell controls not only the inflow rate of Hsp70 into the nucleus but also its outflow rate.

Although we do not yet understand the mechanism of Hsp70 transport into and out of the nucleus, our studies with GFP-Hsp70(K71E) show that this transport involves the ability of Hsp70 to bind ATP tightly or hydrolyze it. In untreated cells, the rates of GFP-Hsp70(K71E) transport into and out of the nucleus were about one-third of the rates observed with GFP-Hsp70. In heat-shocked cells, these rates were even slower so that, in contrast to GFP-Hsp70, GFP-Hsp70(K71E) did not accumulate in the nucleus following heat shock. However, this effect may occur because GFP-Hsp70(K71E) becomes completely immobilized in the nucleus and partially immobilized in the cytosol during heat shock. By contrast, in untreated cells, GFP-Hsp70(K71E) is only slightly less mobile than GFP-Hsp70 and, therefore, its slow rate of transport into and out of the nucleus is probably either because of its inability to bind ATP tightly and hydrolyze it (O'Brien et al., 1996) or because of its related tendency to oligomerize with itself in vitro (Rajapandi et al., 1998) and with Hsp70 in vivo (Hannan et al., 1998). This tendency to oligomerize could also explain why expression of Hsp70(K71E) had a trans-dominant effect on GFP-Hsp70 transport into and out of the nucleus before heat shock and why it prevented these rates from changing during heat shock.

One of the earliest studies on the effect of heat shock on the mobility of Hsp70 suggested that heat shock first induces Hsp70 to bind to nuclear proteins in an ATP-dependent manner and then later induces similar ATP-dependent binding to nucleoli (Lewis and Pelham, 1985). For this reason it was surprising that a recent report on the mobility of GFP-Hsp70 following heat shock found that the mobility of GFP-Hsp70 associated with nucleoli after heat shock was almost the same as that of GFP-Hsp70 free in the cytosol of untreated cells (Kim et al., 2002). By contrast, our results are more in line with the results of the earlier study. GFP-Hsp70, in both the nucleus and the cytosol of untreated cells, has almost the same mobility as GFP, i.e. it is freely diffusable; the slight difference in mobility between GFP-Hsp70 and GFP can be attributed to the higher molecular weight of GFP-Hsp70 as compared to GFP. Even GFP-Hsp70(K71E) appears to be freely diffusable in untreated cells, although it is slightly less mobile than GFP-Hsp70 probably because of its tendency to oligomerize (Newmyer and Schmid, 2001; Rajapandi et al., 1998). On the other hand, we found that, following heat shock, GFP-Hsp70 associated with the nucleoli showed a marked decrease in mobility. In this case, the half-life of fluorescence recovery of GFP-Hsp70 is probably a measure of its rate of dissociation from nucleolar proteins (Coscoy et al., 2002). Interestingly, we found that, following heat shock, even GFP-Hsp70 free in the nucleoplasm showed a marked decrease in mobility, recovering from photobleaching with about a 5 seconds half-life, much slower than GFP alone which recovers in less than 1 second. Therefore, our data show a large difference between the mobility of GFP-Hsp70 free in the cytosol and GFP-Hsp70 bound to unfolded proteins in the nucleolus and nucleoplasm. These differences in mobility of GFP-Hsp70 in the nucleolus of heat-shocked cells and in the cytosol of untreated cells were not detected in the study of Kim et al. (Kim et al., 2002), perhaps because of the rather long time scale they used in determining the rate of recovery after photobleaching. Therefore, our results show that there is one order of magnitude variation in the mobility of GFP-Hsp70 depending on its binding partners.

In confirmation of these data, we found that GFP-Hsp70(K71E) was completely immobilized in the nucleoplasm following heat shock, in contrast to its high mobility before heat shock. Heat shock caused a similar, although less pronounced effect, on GFP-Hsp70(K71E) in the cytosol, although we could detect no change in the mobility of GFP-Hsp70 in the cytosol following heat shock. The remarkable immobility of GFP-Hsp70(K71E) in the nucleus following heat shock suggests that it might bind to the nuclear matrix or chromatin, either directly or indirectly. Although immobilized GFP-Hsp70(K71E) in the cytosol did not seem to follow the distribution of actin filaments or microtubules, microtubules

might be indirectly involved in the immobilization of the GFP-Hsp70(K71E). We found nocodazole increased the mobility of the GFP-Hsp70(K71E) in the cytosol and surprisingly also increased its mobility in the nucleus (data not shown), even though polymerized microtubules are thought not to be present in the nucleoplasm (Walss-Bass et al., 2002; Walss-Bass et al., 2001; Xu and Luduena, 2002). We also found that treatment of heat-shocked cells expressing GFP-Hsp70(K71E) with DTT caused a significant decrease in the amount of immobilized GFP-Hsp70(K71E) in about 25% of the cells (data not shown). Treatment with DTT was previously found to increase the mobility of VSVG-GFP in the endoplasmic reticulum (ER) after ATP depletion or expression of inactive BiP, the Hsp70 homolog in the ER (Nehls et al., 2000). The increase in mobility of GFP-Hsp70(K71E) in DTT-treated cells suggests that, to some extent, formation of disulfide bonds decreases the mobility of GFP-Hsp70(K71E) when it interacts with unfolded proteins during heat shock. Taken together, these data provide direct evidence for ATP-dependent binding of GFP-Hsp70 to unfolded proteins in the cytosol and nucleoplasm following heat shock.

Kim et al. also found that GFP-Hsp70 associated with polyglutamine inclusions had the same mobility as GFP-Hsp70 free in the cytosol (Kim et al., 2002). Based on this result, they argued that polyglutamine inclusions are dynamic structures, despite their observation that GFP-polyglutamine fragments in the inclusions were completely immobilized. By contrast, we find that GFP-Hsp70 associated with polyglutamine inclusions has decreased mobility compared to free GFP-Hsp70, dissociating from the inclusions with a half-life of about 30 seconds, slower than the rate of dissociation of GFP-Hsp70 from nucleoli and more than one order of magnitude slower than the recovery of fluorescence after photobleaching GFP-Hsp70 in the cytosol. Furthermore, GFP-Hsp70 forms shells around the inclusions rather than penetrating them. This effect that has been observed using anti-Hsp70 antibodies for inclusions formed from other proteins (Garcia-Mata et al., 1999), but it remained possible that the antibodies themselves were unable to penetrate the inclusions bodies. However, by using GFP-Hsp70 and doing live imaging, we have now ruled out this explanation and established that Hsp70 indeed forms shells around the inclusions. We have also clearly demonstrated that Hsp70 also forms shells around polyglutamine inclusions in the nucleus. Finally, although we only occasionally found GFP-Hsp70(K71E) associated with polyglutamine inclusions, the associated GFP-Hsp70(K71E) also formed shells around the inclusions and, furthermore, was completely immobilized; its fluorescence did not recover after photobleaching. These observations provide support for the notion that protein aggregates associated with many neurodegenrative diseases may create a substantial sink for Hsp70, perhaps compromising functional capacity and contributing to pathogenesis.

A crucial question about the induction of apoptosis by polyglutamine polymers is whether the toxicity is associated with large insoluble polyglutamine inclusions, or with smaller polyglutamine oligomers or microaggregates present in the cytosol and nucleoplasm (Klement et al., 1998; Marsh et al., 2000; Conway et al., 2000; Wyttenbach et al., 2002). Because Hsp70 protects the cells from the effects of toxic polyglutamine, it is of interest to know whether it only interacts with polyglutamine inclusions or whether it also interacts with the postulated polyglutamine fragments in submicroscopic aggregates. Our results provide evidence that Hsp70 indeed interacts with diffuse polyglutamine aggregates. Although we did not obtain evidence that GFP-Hsp70 interacts with diffuse fragments in the cytosol or the nucleoplasm, we were able to show that GFP-Hsp70(K71E) interacts with diffuse fragments in the nucleoplasm. The half-life for recovery of GFP-Hsp70(K71E) following photobleaching was about 3 seconds in the presence of diffuse polyglutamine fragments, compared to 1.5 seconds in the absence of these fragments. This effect did not occur with Htt(Q25), which strongly suggests that it depends on formation of Htt(Q72) aggregates. We are not certain why the effect occurs only in the nucleus as opposed to the cytosol but one possibility is that numerous transcription factors containing polyglutamine sequences are present in the nucleus but not in the cytosol. Interaction of Htt polyglutamine fragments with these transcription factors could increase the level of aggregation present in the nucleus. As we pointed out above, because Hsp70(K71E) is not dissociated from its binding partners by ATP, its binding is more easily detected than the binding of Hsp70. Nevertheless, it seems possible that the interaction of GFP-Hsp70(K71E) with diffuse polyglutamine fragments reflects an interaction of Hsp70 with these fragments. Therefore, our data provide strong evidence that Hsp70 interacts, either directly or indirectly, with diffuse polyglutamine fragments. This, in turn, is consistent with the theory that diffuse polyglutamine aggregates play a key role in inducing apoptosis of cells in which they are expressed.

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