Overexpression of hsp70i facilitates reactivation of intracellular proteins in neurones and protects them from denaturing stress

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Abstract Transfection of neurones with an adenoviral vector (Ad) expressing high levels of hsp70i was shown to protect primary hippocampal cultures from heat stress. To investigate one of the molecular mechanisms which may underlie hsp70i's neuroprotective effects we measured luciferase activity in the presence and absence of hsp70i following heat or chemical inactivation. Luciferase activity was recovered to 80% of control levels in the presence of recombinant hsp70i in vitro. Luciferase activity was also maintained in primary hippocampal neurones exposed to a denaturing stress if transfected with Ad-hsp70i. These results support the hypothesis that hsp70i protects neurones from stress by interacting with cytosolic proteins and thereby protecting them from inactivation.

Key words: Heat shock protein; Adenovirus; Neuron; Stress; Protein folding

1. Introduction

Cells respond to a sublethal heat shock and several other forms of stress by suppressing normal cellular transcription and translation and at the same time activating the transcription of potentially protective heat shock proteins (hsps). The most abundant of these proteins and the most intensely studied are the constitutively expressed and highly inducible forms belonging to the 70 kDa, hsp70 family. The molecular functions of the constitutively expressed members of the hsp70 family (hsc70s) have been investigated extensively in both eukaryotic and prokaryotic organisms [1,2]. In prokaryotes hsc70 has been shown to increase the yield of native protein in cells either by directly binding and renaturing proteins or by regulating processes to degrade non-native or non-functional proteins [3–7]. In eukaryotes they have multiple functions, acting primarily as intracellular molecular chaperones and as clathrin uncoating enzymes [8]. The inducible forms of hsp70 have been less well studied, though they share a high degree of sequence homology with hsc70 proteins and are therefore also thought to act as molecular chaperones.

Hsp70s have been shown to be monomeric proteins consisting of three domains. An N-terminal domain is the most highly conserved and functions as an ATPase while a C-terminal domain is less highly conserved and is of unknown function. The third domain lies between the N- and C-terminal domains and plays a pivotal role in peptide binding, transport of proteins through intracellular membranes and regulation of the heat shock response [9,10].

Studies of the mammalian brain have shown that induction of hsp70i occurs in animals following epilepsy, ischaemia, trauma, hyperthermia and exposure to excitotoxins [11–13]. Hsp70i immunoreactivity in the hippocampus has been shown to identify neurones destined to survive a transient ischaemic insult with sequential appearance of staining in dentate granule cells and CA3 pyramidal neurones while vulnerable CA1 neurones showed minimal accumulation of hsp70i [14]. Experiments have also shown that induction of a heat shock response can protect cultured neurones from heat, excitotoxic and ischaemic stress [15–17]. In this study we have investigated the mechanism by which the neuroprotective effects of hsp70i may be brought about taking advantage of a highly efficient adenoviral expression system. Using firefly luciferase as reporter protein we have conducted in vitro refolding experiments and investigated the mechanism by which hsp70i interacts with other proteins in a living cell to prevent denaturation following a stress.

2. Materials and methods

2.1. Preparation of rat primary hippocampal cell cultures and Ad infection

Hippocampi were dissected from embryonic rat brains (E15–17) and digested in Hanks' balanced salt solution (HBSS) without calcium and magnesium with 1 mg/ml trypsin for 40 min at 37°C. The cells were washed three times in HBSS and triturated in 500 μl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% B-27 supplement (Gibco BRL), 2% inactivated foetal calf serum (Sigma), glutamine (100 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (this medium is DMEMS). After trituration 2 ml of DMEMS was added and 50 μl of the suspension aliquoted onto 10 mm wells which had previously been coated with polylysine (0.1 mg/ml). After trituration 500 μl DMEMS was added to each well and the final cell concentration was 1×10^6 cells/well. The plates were incubated in 5% CO_2 at 37°C. Cytosine arabinoside (2 μg/ml medium) was added to each well 48 h after plating. Primary hippocampal cultures were transfected at a multiplicity of infection (MOI) of 100, 7 days after plating with the Ad vectors.

2.2. Heat shock and trypan blue staining

Primary hippocampal neurones were heat shocked at 45°C for 20 min. The cells were allowed to recover at 37°C for 48 h. After this period the number of dead cells was determined by trypan blue staining of the cultures. The medium was removed and cells were washed once with phosphate buffered saline (PBS). Cultures were then incubated in trypan blue solution (0.2%) for 5 min and then observed under phase contrast microscopy. Cells were identified as viable by the absence of blue staining within the cell body.

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Abbreviations: CMV promoter, cytomegalovirus major IE promoter enhancer; hsp70i, human inducible heat shock protein; Ad, adenovirus; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolylphosphate; GdmCl, guanidinium hydrochloride

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2.3. Ad vector construction

Recombinant, E1-deleted, Ad constructs were produced according to standard techniques [18–20]. The human hsp70i gene [21] was cloned in the Ad transfer vector pXCXCMV under the control of the human cytomegalovirus major (CMV) IE promoter enhancer fragment (663 bp of pcDNA 1; Invitrogen), followed by a bovine growth hormone poly A tail. The firefly luciferase reporter gene from pGL vector (Promega) was also inserted into a second pXCXCMV. Recombinant viruses were generated by homologous recombination in HEK 293 cells [22] with the helper plasmid pJM17 [23] to create Ad-hsp70i and Ad-luc. Recombinant vectors were grown to high titres and purified by standard techniques [18]. The construction of the regulatable enhanced green fluorescent protein adenoviral construct (Ad-EGFP) has been described previously [19]. Viral titres were determined by plaque assay. In addition Ad-lacZ an Ad encoding the lacZ gene expressing β-galactosidase and Ad-0 a virus with no insert were also used in this study and have been described before [24]. Experiments were conducted at an MOI of 100 which results in approximately 50% of cells being transfected.

2.4. RT-PCR analysis of primary hippocampal neuronal cultures

RNA was isolated (Stratagene) from primary hippocampal cultures and cDNA prepared using AMV reverse transcriptase (Promega). PCR was then performed with primers specific to human hsp70i: sense strand $5' \rightarrow 3'$: GCGGAGAAGTACAAAGCG, antisense strand $5' \rightarrow 3'$: CCCTCCCTTGGGACCCTGA. Reaction products were then separated on a 1% agarose gel and stained with ethidium bromide.

2.5. Western blot analysis

Twenty-four hours post Ad transfection, medium was removed and protein was extracted from the primary hippocampal cultures. The lysate was cleared of cell debris and the protein separated with 10% SDS-PAGE. As standard proteins Bio-Rad rainbow marker was used. The separated proteins were then transferred to a nitrocellulose membrane and hsp70i was detected using anti-hsp70i monoclonal antibody (Stressgen). Blots were then processed using a biotin conjugated secondary antibody (Sigma) and incubated with ExtraAvidin-Alkaline phosphatase (Sigma). The hsp70i bands were visualised using NBT/BCIP solution (Sigma).

2.6. Luminometric assay

To detect luciferase enzyme activity in vitro and in vivo, a luminometric assay was used. In vitro enzyme activity was measured by adding 20 μl of luciferase (0.5 mg/ml) to 100 μl of test substrate (Promega). For in vivo assays neuronal cells were washed in PBS once and lysed in 60 μl cell lysis buffer (Promega). Cells were removed from the well and centrifuged at $12\,000 \times g$ for 1 min. 20 μl of the clear supernatant was added to 100 μl of test substrate. The relative light units of the enzymatic reaction were determined in a Bio Orbit 1253 Luminometer at $\lambda = 562$ nm.

2.7. In vitro luciferase activity assay

To determine luciferase reactivation the enzyme was diluted in buffer A (40 mM Tris-HCl pH 7.7, 2 mM EDTA, 5 mM $MgCl_2$, 5m M ATP, 5 mM DTT (DL-dithiothreitol)). For deactivation 2 mg/ml luciferase was incubated in buffer A plus 7 M GdmCl for 2 h at 25°C. A second method of deactivation was to heat shock 2 mg/ml luciferase in buffer A for 15 min at 41°C. After complete deactivation of luciferase 10 μl of the protein solution was diluted into buffer A (1:100) with or without 20 μg/ml hsp70i. Luciferase reactivation kinetics were measured (described above) immediately after mixing.

2.8. In vivo luciferase activity assay

To determine hsp70i and luciferase activity within neurones hippocampal primary cultures incubated at 37°C and in 5% CO_2 were infected with Ad-luc plus Ad-hsp70i or Ad-luc plus Ad-lacZ. Forty-eight hours after viral transfection with two Ads, hippocampal neurones were heat shocked in an incubator at 42°C, 5% CO_2 for 30 min and then returned to the 37°C incubator. After the heat shock samples were taken at various time points and luciferase activity monitored.

2.9. MTT assays

This assay estimates the metabolic activity of cells by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT, to a coloured formazan product. At each time point 20 μl of MTT (5 mg/ml in PBS) was added to each well of primary hippocampal neurones and incubated for 2 h at 37°C. The medium was then carefully aspirated and 100 μl of acidified isopropanol added to solubilise the coloured formazan product deposited in the viable cells. Absorbance was read at 550 nm on a scanning multiwell spectrophotometer after agitating the plates for 5 min on a shaker.

3. Results

To confirm that the Ad-hsp70i virus could mediate the expression of hsp70i mRNA and protein, RT-PCR and Western blot analysis were used respectively. RT-PCR analysis was carried out on primary hippocampal cultures transfected with Ad-hsp70i, Ad-0 or Ad-lacZ. Transfection with Ad-hsp70i (but not transfection with Ad-lacZ or Ad-0) mediated the transcription of hsp70i mRNA in primary hippocampal cultures (Fig. 1A). Western blot analysis using an anti-hsp70i monoclonal antibody was used to confirm Ad mediated expression of hsp70i protein in hippocampal neurones (Fig. 1B).

Prior to carrying out the reactivation and neuroprotection studies, the efficiency of neuronal transfections was estimated using Ad reporter constructs expressing enhanced green fluorescent protein (Ad-EGFP) and β-gal (Ad-lacZ). Fig. 2A shows that $< 75\%$ of cells could be transfected when primary hippocampal cultures were transfected with the Ad-EGFP (or Ad-lacZ) construct at an MOI of 200. Transfected at MOIs of 100 resulted in 40–50% of cells being transfected [19]. Transfection of cells by Ads entails the binding of the vector to cell surface receptors (predominantly to the Coxsackie adenoviral receptor) followed by endocytosis. The rate of cell transfection

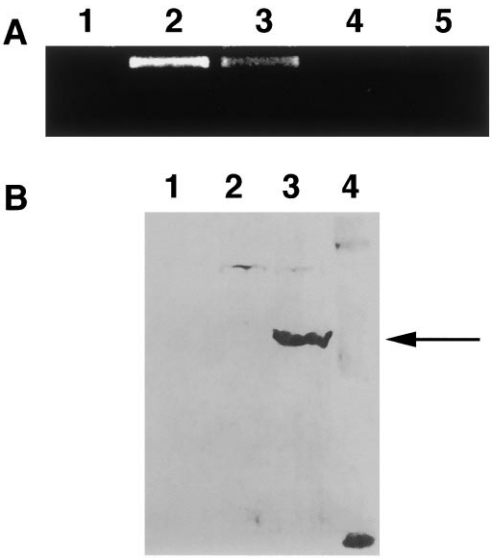

Fig. 1. Detection of hsp70i mRNA and protein following the transfection of primary hippocampal neurones with Ad-hsp70i. A: RT-PCR analysis to detect specific induction of hsp70i mRNA in primary hippocampal neurones transfected with Ad-hsp70i. Lane 1: untransfected primary hippocampal cultures; lane 2: positive control employing a hsp70i encoding plasmid in the PCR; lane 3: primary hippocampal cultures transfected with the Ad-hsp70i virus; lane 4: primary hippocampal cultures transfected with Ad-0 and Ad-lacZ virus respectively. B: Western blot demonstrating the specific induction of hsp70i in primary rat hippocampal neurones transfected with Ad-hsp70i. Lane 1: cells without adenovirus infection; lane 2: infection with Ad-0; lane 3: infection with Ad-hsp70i; lane 4: rainbow marker for SDS-PAGE.

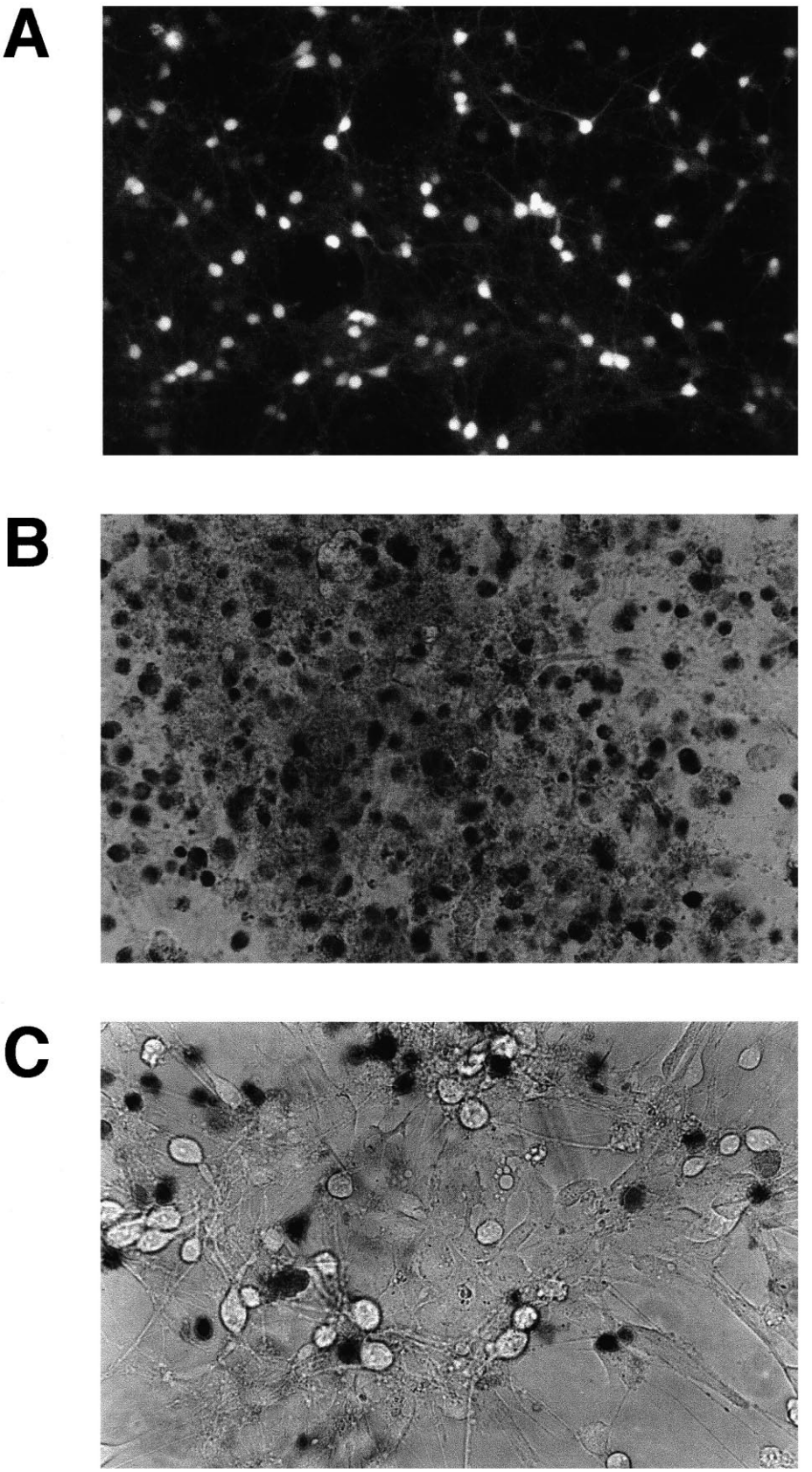

Fig. 2. Protection of primary hippocampal neurones from heat shock following transfection with an Ad expressing hsp70i. A: A fluorescent photomicrograph showing the high number of primary hippocampal neurones transfected with the AdEGFP construct. B and C: Trypan blue staining of cells transfected with the Ad-lacZ and Ad-hsp70i vectors respectively.

is therefore related to the number of virus particles available to bind to its receptors and not to the gene encoded by the construct. In this study to ensure that the same amount of each viral construct was added, the titre of the Ad-hsp70i and Ad-lacZ vectors was measured by plaque assay beforehand and each was diluted to 4.5×10^9/plaque forming (PFU) units/ml.

To investigate the ability of Ad-hsp70i to mediate neuroprotective effects primary rat hippocampal cultures were transfected with either Ad-hsp70i or an Ad-0 vector and then exposed to a sub-lethal heat shock. Cultures transfected with the Ad-hsp70i construct showed a significantly higher cell viability (94% ± 6.0, mean ± S.E.M.) than those transfected with Ad-0 (60% ± 5.0) after heat shock as assessed by trypan blue exclusion assays (Fig. 2). The number of dead cells was greatly underestimated in the Ad-0 transfected cultures as many of the cells had become detached from the surface of the well and these were not counted. Cultures transfected with the constructs not exposed to the heat shock had identical cell viabilities (data not shown).

The ability of hsp70i to increase the rate of reactivation of denatured proteins was studied in vitro and in vivo using luciferase as a reporter protein. Hsp70i's stabilising activity was tested by luminometrically monitoring the enzymatic activity of denatured luciferase in the presence or absence of recombinant hsp70i protein. The results showed that heat or GdmCl inactivated luciferase (data not shown) could be restored to approximately 70% of its former maximal activity in the presence of hsp70i (Fig. 3A). Reactivation of previously heat denatured luciferase was three times higher with hsp70i

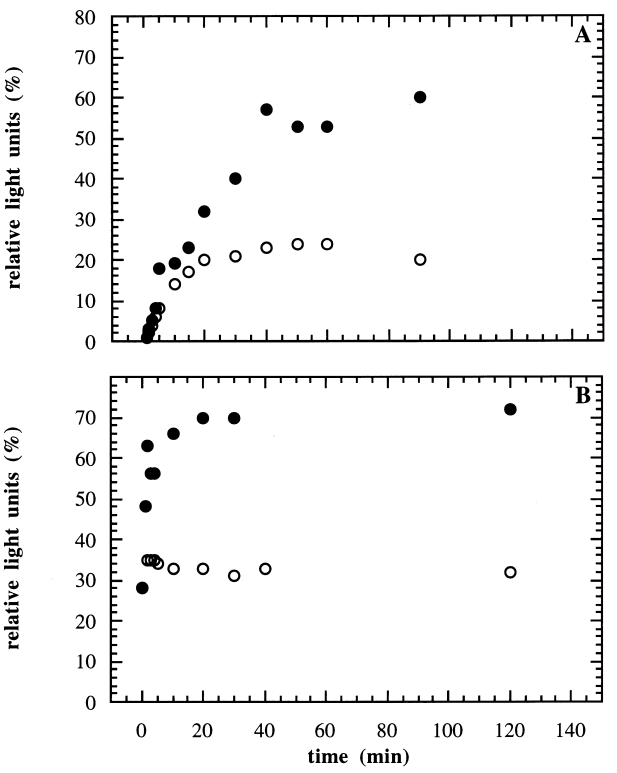

Fig. 3. In vitro reactivation kinetics of (A) GdmCl denatured luciferase (●) with and (○) without hsp70i; (B) thermally denatured luciferase (●) with and (○) without hsp70i. Relative light units refer to native luciferase diluted and assayed in the same way as denatured samples.

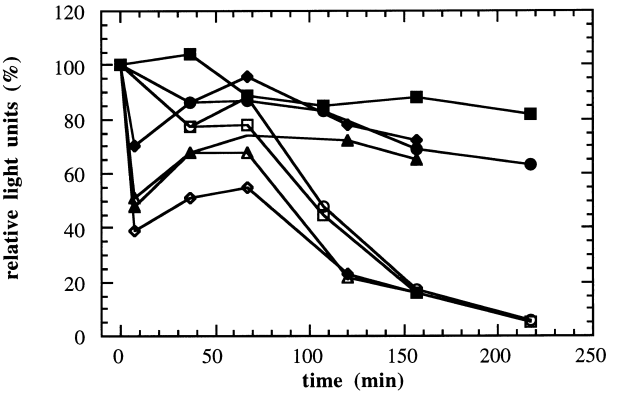

Fig. 4. In vivo protection of luciferase in rat hippocampal primary cultures after heat treatment. Open symbols: luciferase activity in cells transfected with Ad-luc and Ad-lacZ. Closed symbols: luciferase activity in cells transfected with Ad-luc and Ad-hsp70i. Relative light units refer to 100% luciferase activity before heat treatment.

protein added than without the chaperone (Fig. 3B). It was not possible to calculate the respective rate constants as neither reaction followed first or second order kinetics. To determine whether overexpression of hsp70i could also prevent a decrease in luciferase activity in vivo primary hippocampal neurones were transfected with Ad vectors expressing hsp70i and luciferase and then exposed to a heat stress. In control experiments Ad-hsp70i was replaced with an Ad expressing β-galactosidase. The fact that neuronal cells can be successfully transfected by two Ad vectors at the same time has previously been shown by Harding et al. [19,20]. Higher levels of luciferase activity were observed in cells transfected with Ad-hsp70i plus Ad-luc when compared to cells transfected with Ad-luc plus Ad-lacZ (Fig. 4). In the presence of Ad-hsp70i luciferase activity did not significantly decrease and remained at approximately 80% of maximum activity for at least 2 h after heat shock. In contrast cells transfected with Ad-luc plus Ad-lacZ showed a decrease in luciferase activity with time and 2 h following the heat shock luciferase activity was no longer detectable. The luciferase activity prior to heat shock was identical in Ad-hsp70i transfected and Ad-lacZ transfected cultures and set as 100%.

MTT assays were also carried out to compare the relative toxicities of the transfection and heat shock procedures. These experiments clearly showed that 24 h after heat shock (and 48 h after transfection) neurones which were transfected with Ad-hsp70i and then exposed to a heat shock had significantly higher mitochondrial activity (as estimated by the MTT assay) than cells transfected with Ad-EGFP or Ad-lacZ and then heat shocked. MTT values ± S.E.M. were; Ad-hsp70+heat shock (HS) = 3.3 ± 0.075; Ad-EGFP+HS = 2.6 ± 0.1; Ad-lacZ+HS = 2.65 ± 0.05. $P < 0.0001$ when Ad-hsp70+HS was compared to the Ad-EGFP or Ad-lacZ experiments by Student's *t*-test.

4. Discussion

This and other recent studies [19,20,24,25] have shown that E1-deleted type 5 adenoviruses are a powerful new molecular tool which should greatly facilitate studies into neuronal gene function. The data in this investigation show that Ad mediates the highly efficient transfection of primary hippocampal neu-

rones, and that Ad mediated overexpression of hsp70i can enhance the survival of neurones following exposure to severe heat stress. These results support the findings of Rodorf et al. [15] and Lowenstein et al. [17] who showed that exposure of primary neuronal cultures to a sub-lethal elevation in temperature induced hsp expression and protected these cells against a subsequent stress. We have also shown that recombinant hsp70i protein alone can mediate the reactivation of denatured luciferase in vitro. Furthermore, Ad mediated luciferase enzymatic activity in neurones is only maintained at high levels following a heat stress when the cells are also co-transfected with Ad-hsp70i. Following heat stress, denatured protein such as luciferase may aggregate and/or bind non-specifically to cellular proteins interfering with their normal cellular function [26]. Our results suggest that hsp70i is interacting directly with denatured luciferase and supports the hypothesis that hsp70i may protect cells against stress which results in protein denaturation and aggregation by allowing them to stabilise and reactivate partially denatured proteins.

Recent work has shown that some hsc70 proteins may be directed to different protein substrates by interacting with proteins known collectively as DnaJs or hsp40s [27,28]. Several eukaryotic DnaJ homologues have now been isolated suggesting that DnaJ proteins may participate in at least some of the reactions catalysed by eukaryotic hsp70 family members [29]. Cheetham et al. [30] isolated a human alternatively spliced DnaJ homologue (hsj1a and hsj1b) and showed that the isoforms were stress inducible and located exclusively in the brain. Another mammalian DnaJ homologue isolated from HeLa cells, hsp40, has been shown to be highly stress inducible and accumulates in the nucleus and nucleolus during stress, a pattern similar to that seen for hsp70i. In addition hsp40 was co-localised with hsc73 in heat shocked HeLa cells. These results were interpreted as suggesting that hsp40 and hsc70/hsp70i may act together to prevent nascent proteins from becoming abnormally folded and may also prevent protein aggregates from accumulating [31,32]. A more recent study has shown that the overexpression of HSDJ (a hsp40 chaperone protein which interacts with hsp70 proteins to mediate protein refolding and translocation) prevented the accumulation of ataxin-1 in a transgenic mouse model of spinocerebellar ataxia type 1 (SCA1) [33]. These findings suggest that increasing the expression of DnaJ like proteins in vivo may also facilitate the neuroprotective effects of hsp70i. Indeed, it may be that hsp70i acts to some degree with constitutively expressed DnaJ proteins in vivo to mediate its protective effects. The presence of insoluble protein aggregates, such as those found in SCA1, is a hallmark of a number of other neurodegenerative diseases, e.g. Lewy bodies in Parkinson's disease, neurofibrillary tangles and amyloid plaques in Alzheimer's disease. The mechanism which leads to protein aggregation is unknown but it is possible that the disease itself is caused by abnormal protein-protein interactions which eventually results in the accumulation of insoluble protein aggregates. Alternatively, it may be that aberrant insoluble proteins are produced during the disease progress due to altered protein metabolism. The data presented in this study suggest that the overexpression of chaperone molecules in neurones stabilises and maintains the function of intracellular proteins and thus slows or prevents inactivation due to stress. Therefore, the strategy of inducing the expression of specific chaperone molecules to delay the onset of neurodegenerative disease, especially those characterised by the accumulation of abnormal proteins should be investigated.

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