Hsp40 Molecules That Target to the Ubiquitin-proteasome System Decrease Inclusion Formation in Models of Polyglutamine Disease

JL Howarth[1], S Kelly[1], MP Keasey[1], CPJ Glover[1], Y-B Lee[1], K Mitrophanous[2], JP Chapple[3], JM Gallo[4], ME Cheetham[3] and JB Uney[1]

[1]Henry Wellcome Laboratories for Integrated Neuroscience and Endocrinology, University of Bristol, Bristol, UK; [2]Oxford BioMedica (UK), Medawar Centre, The Oxford Science Park, Oxford, UK; [3]Department of Molecular and Cellular Neuroscience, Institute of Ophthalmology, University College London, London, UK; [4]Department of Neurology, Institute of Psychiatry, King's College London, London, UK

We studied the ability of heat shock, DnaJ-like-1 (HSJ1) proteins (which contain DnaJ and ubiquitin-interacting motifs) to reduce polyglutamine-mediated inclusion formation. The experiments demonstrated that expression of heat shock protein 70 (hsp70), hsp40, HSJ1a, and HSJ1b significantly reduced protein inclusion formation in a model of spinal and bulbar muscular atrophy (SBMA). HSJ1a also mediated a significant decrease in the number of inclusions formed in a primary neuronal model of protein aggregation. Studies to elucidate the mechanisms underlying these reductions showed that hsp70 and hsp40 increased chaperone-mediated refolding. In contrast, expression of HSJ1 proteins did not promote chaperone activity but caused an increase in ubiquitylation. Furthermore, HSJ1a was associated with a ubiquitylated luciferase complex, and in the presence of HSJ1a but not an HSJ1a UIM mutant (HSJ1a-ΔUIM) there was a reduction in luciferase protein levels. Together these results show that HSJ1 proteins mediated an increase in target protein degradation via the ubiquitin-proteasome system (UPS). We also found that the expression of HSJ1a significantly decreased the number of neurons containing inclusions in an *in vivo* model of polyglutamine disease. These findings indicate that targeted modification of the UPS to facilitate degradation of misfolded proteins may represent a highly effective therapeutic avenue for the treatment of polyglutamine disease.

Received 21 November 2006; accepted 5 March 2007; published online 10 April 2007. doi:10.1038/sj.mt.6300163

INTRODUCTION

The polyglutamine disorders, such as spinal and bulbar muscular atrophy (SBMA) and Huntington's disease, are characterized by the inexorable dysfunction and death of neurons. The disorders are caused by an expansion in the unstable glutamine trinucleotide (CAG) repeat region of affected genes.[1,2] It is known that the CAG expansion confers a toxic gain-of-function and results in the translation of a mutant protein that aggregates to form inclusions within the affected neurons.[3–5] Importantly, an early age of disease onset and increased disease severity are directly correlated with increasing length of the polyglutamine expansion and hence increased propensity of the mutant protein to aggregate. The mechanism by which the mutant protein mediates neuronal cell death remains uncertain, however. Insoluble nuclear or cytoplasmic protein aggregates and/or inclusions were initially thought to activate apoptotic pathways and/or alter patterns of gene transcription,[6–12] but it has been suggested that monomeric or small oligomers of mutant protein may be responsible for neuronal toxicity and that insoluble inclusions may be protective as they sequester the deleterious forms of mutant protein.[13–16]

Intracellular polyglutamine-containing inclusions have been found to be associated with small heat shock protein (hsp), ubiquitin, and hsp70. These observations suggest that hsps are associated with the etiology of these diseases, or more likely that their presence represents an attempt to refold or remove the abnormal protein aggregates. Consistent with this hypothesis is the finding that the over-expression of chaperones suppresses inclusion formation and cell death.[17–19] Typically, studies have investigated the actions of hsp70 and its co-chaperone hsp40. Hsp40 co-chaperones are positive regulators that facilitate refolding of substrate proteins by enhancing the adenosine triphosphatase activity of hsp70 family members.[20,21] In contrast, negative regulators compete with co-chaperones and consequently convert hsp70 from a protein-refolding machine into a factor that targets the substrate protein to the ubiquitin-proteasome system (UPS).[22] Bcl-2-associated athanogene-1 (Bag-1)[23] proteins and the E3 ligase carboxyl-terminus of hsp70-interacting protein (CHIP)[24] are examples of negative regulators, and they form an important link between the chaperone and ubiquitin-protein degradation systems.[25–30] Importantly, recent studies have shown that Bag-1 and CHIP associate with polyglutamine aggregates[31–33] and assist in their degradation.

Recently, two neuronally enriched members of the hsp40 family termed HSJ1a and HSJ1b, which may act as another link between the molecular chaperone system and the UPS, were

Correspondence: *J.B. Uney, University of Bristol, Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, UK. E-mail: james.uney@bristol.ac.uk*

identified.[34] HSJ1 proteins have distinct intracellular localizations: HSJ1a is cytoplasmic, whereas the larger HSJ1b isoform is targeted to the cytoplasmic face of intracellular membranes.[35] Both proteins can regulate the adenosine triphosphatase activity and substrate binding of hsp70. Moreover, HSJ1a and HSJ1b contain ubiquitin-interacting motifs (UIMs) that bind ubiquitylated proteins and target them to the proteasome.[31] At present, it is not known under what circumstances these proteins act as co-chaperones or mediate proteasomal sorting.

In this study we have used adenoviral (Ad) gene delivery systems with *in vitro* and *in vivo* models of SBMA and polyglutamine expansion diseases to study the function of HSJ1 proteins. The results show that in our models HSJ1 proteins did not promote hsp70 chaperone activity but were highly effective at reducing aggregation *in vitro* by increasing protein ubiquitylation and targeting to the UPS. Furthermore, polyglutamine-mediated inclusion formation *in vivo* was dramatically reduced when neurons were transduced with a lentivirus expressing HSJ1a.

RESULTS

Studying chaperone function using an SBMA neuronal cell culture model

Experiments were conducted in N2a cells transfected with constructs that contained the ligand binding domain of human androgen receptor (hAR) and a glutamine repeat fragment of either 20 or 51 repeats.[36,37] Cells were treated with testosterone and an antibody against the androgen receptor used to detect the expression of wild-type hAR (containing a 20-glutamine repeat fragment) and "expanded" hAR knock-in (hARk) (containing 51 repeats). Immunofluorescence showed that hAR was localized within the nucleus of N2a cells, whereas hARk formed inclusions that accumulated within the cytoplasm (**Figure 1a**). To investigate the ability of chaperone molecules to reduce inclusion formation, advectors were used to express hsp70i, hsp40, HSJ1a, and HSJ1b in N2a cells. Western blots confirmed Ad-mediated expression of each chaperone (**Figure 1b**), and cells transduced with an Ad control vector (Ad0) showed no chaperone expression above background. Ad-mediated expression of the inducible form of hsp70 (hsp70i) was found to mediate a significant decrease ($P < 0.01$) in the percentage of cells containing cytosolic polyglutamine inclusions (**Figure 1c**). Expression of hsp40/Hdj1 alone also mediated a significant decrease ($P < 0.001$) in the percentage of cells containing polyglutamine inclusions; however, co-expression with hsp70 did not result in a further reduction in inclusions (**Figure 1c**). The Ad-mediated expression of both HSJ1a and HSJ1b also brought about a statistically significant decrease ($P < 0.001$) in the percentage of cells containing polyglutamine inclusions (**Figure 1c**).

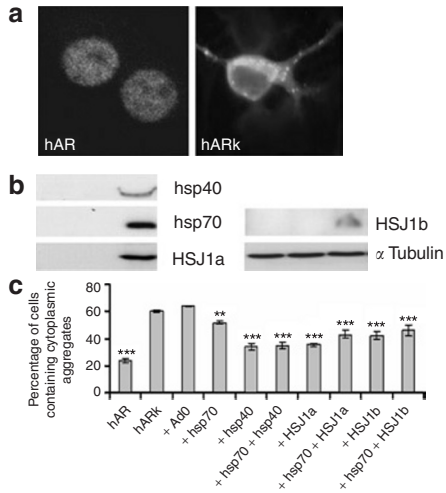

Figure 1 Studying inclusion formation in a spinal and bulbar muscular atrophy neuronal cell culture model. (**a**) N2a cells were transfected with wild-type human androgen receptor (hAR) or "expanded" human androgen receptor knock-in (hARk) constructs, and the formation of cytoplasmic inclusions after the administration of testosterone was observed by immunofluorescence light microscopy. (**b**) Cells were transduced with adenoviral (Ad) vectors, and the expression of heat shock protein 70 (hsp70), hsp40, heat shock, DnaJ-like-1a (HSJ1a), and HSJ1b was verified by western blotting (lane 3 in each blot). Lanes 1 and 2 are untransduced cells and cells transduced with an adenoviral control vector (Ad0), respectively. (**c**) Cells were transfected with the hARk constructs, and the number of cells containing cytosolic polyQ aggregates was counted after transduction with vectors expressing hsp70, hsp40, HSJ1a, or HSJ1b. The results are represented as the percentage of cells containing aggregates relative to the untreated control + SEM ($n = 12$). Chaperone-transduced cells were compared with the hARk control, and statistical analysis was carried out by analysis of variance followed by post hoc *t*-tests. ***$P < 0.001$, **$P < 0.01$.

Estimating chaperone activity using luciferase-refolding assays

To obtain a quantitative measure of chaperone activity, luciferase-refolding assays were used. N2a cells were transduced with Ad vectors expressing luciferase and chaperone molecules, and after 3 days the cells were heat-shocked to mediate thermal denaturation of the luciferase. The results showed that when expressed alone, hsp70 moderately increased denatured luciferase activity, whereas hsp40 had no detectable effect (**Figure 2a**); however, expression of HSJ1a and HSJ1b caused a reduction in luciferase activity. When cells were simultaneously transduced with hsp70 and hsp40, a statistically significant increase in luciferase activity was observed. In contrast, transduction with Ad vectors mediating the simultaneous expression of hsp70 plus HSJ1a or hsp70 plus HSJ1b did not facilitate the recovery of denatured luciferase activity (**Figure 2b**). When luciferase activity was measured after transduction with the Ad HSJ1a-ΔUIM vector (which cannot mediate proteasomal sorting[34]), no recovery in luciferase activity was seen following denaturation. Expression of HSJ1a-ΔUIM plus hsp70 mediated a small but non-significant increase in luciferase activity, whereas expression of hsp70 plus hsp40 again mediated a significant recovery in luciferase activity (**Figure 2c**).

HSJ1 proteins mediate protein-ubiquitylation of luciferase

Experiments to investigate steady-state luciferase activity in the presence of molecular chaperones showed that the over-expression of HSJ1a and HSJ1b caused a highly significant decrease in the steady state of luciferase activity in the N2a cells (**Figure 3a**). However, transduction with an Ad vector expressing HSJ1a with deletions in the UIM (Ad-HSJ1a-ΔUIM) did not mediate a decrease in steady-state luciferase activity. In addition, the over-expression of hsp40 and hsp70i (either alone or in combination) did not reduce steady-state luciferase activity. To

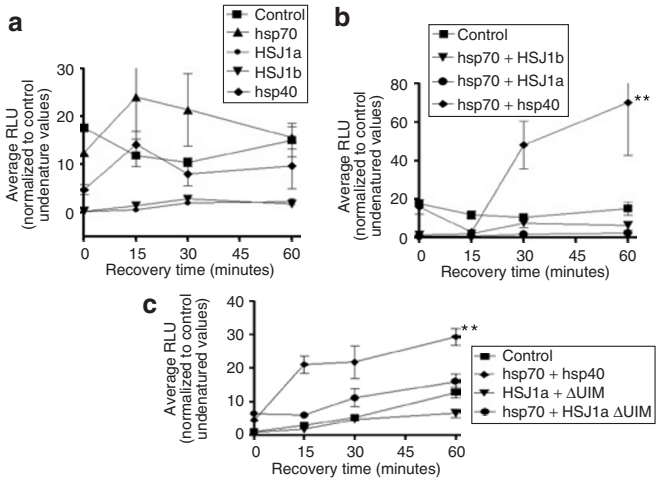

Figure 2 Luciferase refolding mediated by chaperones and co-chaperone molecules. N2a cells were transduced with an adenoviral (Ad) vector expressing luciferase and luciferase activity following a heat stress measured in the presence of (**a**) heat shock protein 70 (hsp70), heat shock, DnaJ-like-1a (HSJ1a), HSJ1b, and hsp40; (**b**) hsp70 co-expressed with HSJ1b, HSJ1a, or hsp40; or (**c**) hsp70 co-expressed with hsp40 or a mutant HSJ1a (HSJ1a-ΔUIM) that contained deletions in the UIMs making them non-functional. $^{**}P < 0.01$ when compared by analysis of variance followed by post-hoc *t*-tests with controls ($n > 3$). RLU, relative light unit.

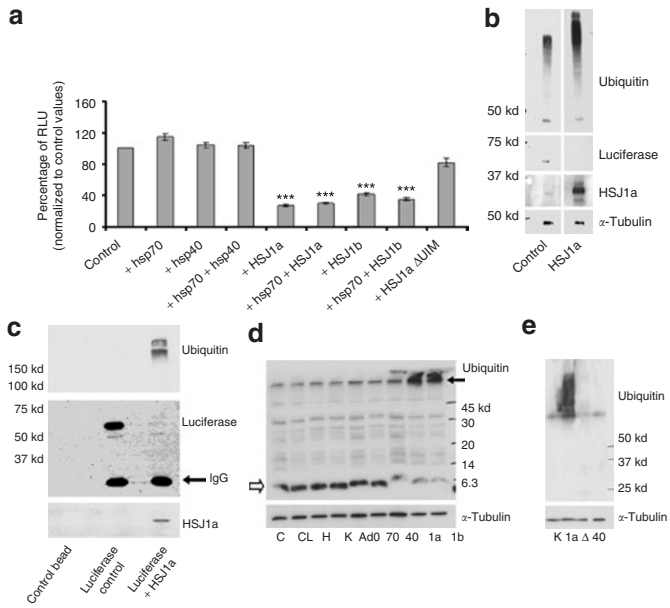

Figure 3 Heat shock, DnaJ-like-1 (HSJ1) proteins caused a reduction in steady-state luciferase activity and mediated the ubiquitylation of target proteins. (**a**) Analysis of steady-state (non-heat-denatured) luciferase values in N2a cells in the presence of heat shock protein 70 (hsp70), hsp40, HSJ1a, HSJ1b, and HSJ1a-ΔUIM (expressed either alone or in combination). (**b**) Western blots of control cells and N2a cells transduced with HSJ1a and luciferase and probed with anti-ubiquitin, anti-luciferase, and anti-HSJ1a antibodies. (**c**) Luciferase co-immunoprecipitation assays in the presence of HSJ1a showing that ubiquitin and HSJ1a are bound with luciferase. (**d**) Western blots of ubiquitin in human androgen receptor knock-in (hARk)–expressing N2a cells transduced with hsp70, hsp40, HSJ1a, or HSJ1b. The black arrow indicates the retardation of the ubiquitin-labeled species at the top of the gel. α-tubulin was used as a loading control. (**e**) Western blots of hARk-transfected N2a cells expressing HSJ1a, HSJ1a-ΔUIM, and hsp40. IgG, immunoglobulin G; RLU, relative light unit.

investigate whether HSJ1a was mediating a decrease in luciferase levels rather than activity, western blots of luciferase and HSJ1a–transduced cells were carried out (**Figure 3b**). The results showed that luciferase protein was detected after transduction with Ad-cytomegalovirus luciferase; however, the simultaneous expression of HSJ1a resulted in luciferase protein being undetectable. Further blot analysis of luciferase pull-down assays showed that ubiquitin was immunoprecipitated with luciferase and that HSJ1a was associated with this complex (**Figure 3c**). Western blots were then carried out on N2a cells that had been transfected with hAR and hARk plasmids and transduced with Ad vectors expressing chaperone molecules. These experiments showed that transduction with HSJ1a or HSJ1b, but not other chaperone molecules, led to a shift in ubiquitin staining. After the expression of the HSJ1 proteins, ubiquitin was bound to high-molecular-weight proteins, as demonstrated by the retardation of ubiquitin at the top of the gel, presumably bound to large protein complexes or insoluble protein inclusions (**Figure 3d**). The level of free mono-ubiquitin (approximately 8.5 kd) was also lower in these cells than in control cells and cells transfected with hsp70. Further experiments using Ad vectors expressing HSJ1a and HSJ1a-ΔUIM showed that the increase in ubiquitylation in hARk-transfected cells was mediated via the UIM domains (**Figure 3e**).

Effect of HSJ1a and HSJ1b expression on Q80EGFP aggregation in primary cortical neurons

Neurons were transduced with Ad vectors expressing Q19EGFP and Q80EGFP fusion proteins under the control of a neuron-specific promoter (**Figure 4a**). Transduction of cortical neurons with an Ad vector expressing Q19EGFP caused diffuse enhanced green fluorescent protein (EGFP) staining throughout neurons, whereas the Ad vector expressing Q80EGFP produced distinct nuclear inclusions (**Figure 4b**). In addition, transduction of primary cortical neurons expressing the polyQ expansion with HSJ1a led to a highly significant decrease ($P < 0.001$) in insoluble Q80EGFP (**Figure 4c**). HSJ1b also mediated a significant but smaller decrease in insoluble Q80EGFP. In comparison, transduction with hsp70 or hsp40 did not mediate a significant decrease in the presence of insoluble aggregates (**Figure 4c**).

HSJ1a prevents polyglutamine-mediated aggregation in the striatum

Lentiviral vectors expressing Q19EGFP and Q80EGFP fusion proteins under the control of the neuron-specific synapsin promoter were injected into the striatum, and the formation of inclusions was monitored. The injection of the control vector (HIV-1-Syn1-Q19EGFP-WPRE) did not result in the formation of EGFP-positive inclusions, but produced very faint, diffuse EGFP staining throughout the whole cell (**Figure 5a**). In contrast, intrastriatal injection of the "expanded" vector (HIV-1-Syn1-Q80EGFP-WPRE) led to the widespread formation of nuclear inclusions. Statistical analysis of these data demonstrated that there were more cells containing EGFP-positive inclusions at 21 ($P < 0.05$) and 35 days ($P < 0.01$) than at 7 days (**Figure 5b**). However, there was

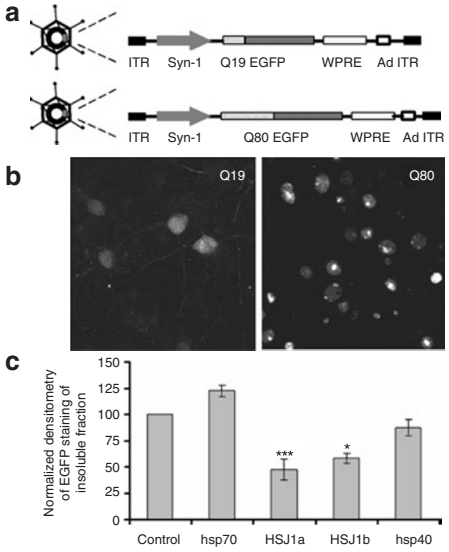

Figure 4 Heat shock, DnaJ-like-1 (HSJ1) protein expression prevents inclusion formation in primary neurons expressing an expanded polyglutamine (Q80) region linked to enhanced green fluorescent protein (EGFP). (**a**) Schematics of QnEGFP adenoviral vectors used to transduce primary cultures showing the CAG fragment (Q19 or Q80) driven by the Synapsin-1 promoter and enhanced by the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). (**b**) Transduction of primary cortical neurons with adenoviral (Ad) vectors expressing Q19EGFP or Q80EGFP. (**c**) Cortical neurons were transduced with Ad vectors expressing Q80EGFP in the presence of heat shock protein 70 (hsp70), hsp40, and HSJ1 proteins, and filter-trap assays using a GFP antibody were carried out to observe the formation of insoluble aggregates.

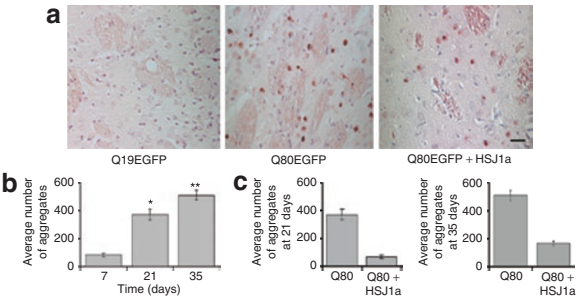

Figure 5 Heat shock, DnaJ-like-1a (HSJ1a) reduced polyglutamine-mediated inclusion formation in the striatum. (**a**) Lentiviral vectors expressing Q19EGFP, Q80EGFP, and Q80EGFP plus HSJ1A were injected into the striatum, and hematoxylin and eosin–stained sections were probed with an anti–enhanced green fluorescent protein (EGFP) Ab. Images were taken at ×200 and the scale bar represents 20 μm. (**b**) Counts of EGFP-positive aggregates in the striatum 7, 21, and 35 days after the injection of a lentivirus expressing Q80EGFP, and (**c**) on animals injected with vectors expressing HSJ1a and Q80EGFP 21 and 35 days after injection. Statistical analysis was carried out by one-way analysis of variance and post hoc *t*-tests. Error bars are SEM. $n = 5$. $^*P < 0.05$, $^{**}P < 0.01$ when compared with controls.

no significant difference between the numbers of cells containing inclusions at 21 and 35 days. When a lentivirus expressing HSJ1a was injected simultaneously with Q80EGFP, a dramatic decrease in cells containing EGFP-positive inclusions at both 21 ($P < 0.05$) and 35 days ($P < 0.05$) was observed (**Figure 5a** and **c**).

DISCUSSION

Our results show that when expressed alone, hsp70, hsp40, HSJ1a, and HSJ1b were effective at preventing the accumulation of inclusions in an *in vitro* model of SBMA. An additional, simplified polyglutamine expansion model, using the viral expression of EGFP fused to polyQ repeat tracts of 19 or 80 CAG repeats, showed that these chaperone molecules mediated the same effects in primary neurons. We also demonstrated that a significant reduction in inclusion formation was mediated by the expression of HSJ1 proteins, whereas hsp40 expression mediated a small but non-significant reduction in inclusion levels. Luciferase-refolding assays conducted in N2a and primary cortical neurons (data not shown) showed that hsp70 and hsp40 significantly increased chaperone-mediated luciferase refolding. However, the increased expression of HSJ1 proteins or HSJ1a-ΔUIM proteins (containing mutated and non-functional UIM domains) did not increase luciferase refolding. Indeed, the expression of HSJ1a and HSJ1b actually caused a dramatic reduction in steady-state (non-denatured) luciferase activity together with increased protein ubiquitylation within cells. In contrast, no reduction in luciferase activity and increased protein ubiquitylation was observed with the HSJ1a-ΔUIM construct. Experiments also showed that the reduction in luciferase activity was associated with a reduction in its protein levels and that luciferase was found with an HSJ1a–ubiquitin complex. Neither HSJ1a nor the DnaJ-containing UIM-mutated HSJ1a mediated significant refolding activity.

Together these results show that within neuronal cells HSJ1 proteins do not reduce inclusion formation by facilitating the folding function of hsp70. Instead, we showed that HSJ1a mediated increased ubiquitylation and a reduction in (luciferase) protein and polyglutamine-mediated inclusion levels. These findings strongly suggest that HSJ1a is mediating increased polyglutamine protein ubiquitylation and targeting to the UPS. Importantly, *in vivo* studies also showed that the lentiviral-mediated expression of HSJ1a dramatically decreased polyglutamine-mediated inclusion formation in the striatum.

Studies have shown that the increased expression of protein chaperones can decrease the neuronal toxicity and/or accumulation of protein inclusions associated with polyglutamine disease.[17–19] In this study we have investigated the ability of the human HSJ1a and HSJ1b proteins to reduce inclusion formation in two models of polyglutamine expansion diseases. Both proteins are preferentially expressed in neurons and display distinct intracellular localizations. HSJ1a is cytoplasmic and nuclear, whereas the larger HSJ1b isoform is normally targeted to the cytoplasmic face of the endoplasmic reticulum by C-terminal geranylgeranylation. HSJ1a and HSJ1b both contain UIMs and also belong to the family of J-proteins that stimulate adenosine triphosphate hydrolysis by heat shock cognate 70 (hsc70). UIMs play an important role in mediating ubiquitylation and in binding mono- and/or polyubiquitylated proteins and targeting to the UPS. In recent years it has become apparent that the UPS plays a pivotal role in regulating and maintaining protein homeostasis in cells and that under some circumstances molecular chaperones can target substrate proteins to the UPS for degradation.[28] An example of chaperone-mediated targeting is provided by CHIP that uses hsp70 and hsp90 family members to recognize misfolded protein substrates, which it then directs for degradation via the UPS. The function of CHIP is enhanced by Bag-1, another co-chaperone

that simultaneously associates with hsc70.[27] On formation of the Bag-1/hsc70/CHIP complex, CHIP mediates the attachment of ubiquitin moieties to the ubiquitin-like domain found on all Bag-1 isoforms.[25,38] This domain enables Bag-1 to function as a physical link (or coupling factor) between the hsc70/hsp70 chaperone system and the proteasome.

As HSJ1 proteins contain DnaJ and UIMs, they could act to mediate chaperone function or fulfill a similar role to Bag-1 proteins (acting as a switch between chaperone refolding activities of hsc70 and its targeting to the UPS).

In this study we have shown that in the presence of insoluble polyglutamine inclusions, HSJ1a acts via its UIM domains to initiate degradation of abnormal proteins rather than their refolding. Indeed, even when the UIM domain was deleted there was no significant increase in chaperone activity. Hence, these findings show that DnaJ/hsp40 proteins (*e.g.*, hsp40 and HSJ1 molecules) can be used to reduce the level of polyglutamine-mediated aggregation by enhancing refolding and by increasing ubiquitylation of the substrate protein. The effectiveness of HSJ1a in preventing inclusion formation in models of neuronal polyglutamine expansion diseases suggests that targeting to the UPS may be a highly therapeutic strategy.

MATERIALS AND METHODS

Transfection of androgen receptor. Mouse neuroblastoma (N2a) cells were grown in Dulbecco's minimum essential medium with 5,000 mg/l glucose (Sigma, Dorset, UK), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Gibco-Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mmol/l L-glutamine (both Sigma, Dorset, UK) in a humidified 5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 5,000–20,000 cells per well onto presterilized glass coverslips. After 48 hours cells were transfected with pCMVneo-based expression vectors containing either a 20-glutamine repeat fragment (wild-type, hAR) or an expanded polyglutamine coding region with 51 repeats (hARk), using lipofectamine reagent (Invitrogen, Karlsruhe, Germany).[37] Cells were transduced with virus 12 hours after transfection (as described in the Results), and after a further 12 hours, cells were transferred to phenol red–free medium supplemented with 10% dextran-coated, charcoal-stripped fetal calf serum (Hyclone, Perbio Science, Cheshire, UK), before incubation with 50 nmol/l testosterone and subsequent assays.

Primary cortical neuron cultures. Primary cortical tissue was dissected from Wistar rat embryos at embryonic day 18 and placed into Hanks' balanced salt solution without calcium and magnesium (Gibco-Invitrogen, Karlsruhe, Germany) with 10 mg/ml trypsin (Sigma, Dorset, UK) for 30 minutes at 37 °C. The cells were then washed three times in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and triturated gently in Neurobasal medium (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 2% (vol/vol) B-27 supplement (Gibco-Invitrogen, Karlsruhe, Germany), glutamic acid (25 μmol/l), L-glutamine (0.5 mmol/l), streptomycin (100 μg/ml), and penicillin (100 U/ml) (all from Sigma, Dorset, UK) using a glass pipette with a fire-polished tip. Cells were plated at a density of 170,000 cells into each well of a 24-well plate (Nalge Nunc International, UK) that had previously been coated with poly-L-lysine (1 mg/ml; Sigma, Dorset, UK). After 3 days one-third of the culture medium was replaced with Neurobasal medium supplemented with B-27, penicillin, and streptomycin. Cultures were transduced with Ad vectors after 5 days, with further medium changes every 3 days. All animal work was performed under a Home Office license and under the supervision of staff at the central animal facility, University of Bristol.

Virus production. E1-deleted Ad vectors expressing various hsps and co-chaperones were produced by homologous recombination in HEK 293 cells according to standard techniques.[39,40] Expression was driven by the human cytomegalovirus promoter and enhanced by the woodchuck hepatitis virus post-transcriptional regulatory element. These viruses were used to transduce N2a cells expressing the hARk plasmid. Additional Ad vectors expressing CAG repeat fragments tagged with GFP (QnEGFP) from the neuron-specific synapsin promoter were also used to transduce primary cortical neuron cultures. Vesicular somatitis virus-glycoprotein–pseudotyped lentiviral vectors expressing Q19EGFP, 80EGFP, HSJ1a, and HSJ1b were prepared using the HEK 293T transient system previously described,[41] and viral titer was determined by reverse-transcriptase polymerase chain reaction.

Stereotactic injection. Intrastriatal injections were performed bilaterally on isofluorane-anesthetized adult male Sprague-Dawley rats (300–380 g) that had been placed in a stereotactic frame (David Kopf Instuments, CA). The stereotactic coordinates for all intra–central nervous system injections were derived from Paxinos and Watson[42] and were from bregma: anterior–posterior, 0 mm; medial–lateral, ±3.0 mm; and dorsal–ventral, –4–5 mm. Generally, 1.5×10^6 transforming units in a total volume of 1 μl of each lentiviral vector were slowly infused into the striatum, with ratios kept constant. Thus, approximately equal numbers of Q80 and HSJ1a viral particles were injected. At the times indicated after injection, the brains were embedded in paraffin and sectioned at 7 μm on a microtome (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry on wax-embedded sections. After dehydration of the sections in alcohols, antigenic site retrieval was achieved by microwaving them in citric acid buffer (pH 6.0) before washing with 1× phosphate-buffered saline supplemented with 0.5% Trition X-100 and 1% H_2O_2 in phosphate-buffered saline. Sections were then blocked with normal horse serum (VectorLabs, UK), and incubated with anti-GFP antibody (Roche) at 4 °C overnight. Sections were then incubated with biotinylated anti-mouse IgG secondary antibody (VectorLabs) and streptavidin/biotin/peroxidase conjugate (ABC Elite; VectorLabs, UK). Nova Red stain (VectorLabs) was used to visualize positive cells. Sections were counterstained with hematoxylin and eosin, and images were taken using a Leica upright microscope (Leica Microsystems, Wetzlar, Germany). Microsoft Office Document Imaging software (Microsoft Corporation) was used to count cells that contained EGFP-positive inclusions. All cell counts were performed blind.

Immunofluoresence microscopy. After fixation in methanol, transfected cells were incubated with anti-androgen receptor antibody, AR N20 (1:150; Santa Cruz Biotechnology, CA). Fluorescein isothiocyanate–conjugated anti-rabbit antibody diluted 1:200 was then used to visualize inclusions within the cells. Cells were mounted with non-quenching medium (Vectashield; Vectorlabs, CA); images were captured using an Inverted Leica Confocal Imaging system (Leica Microsystems, Wetzlar, Germany), and counts were performed blind.

Luciferase-refolding assay. N2a cells were transduced with Ad vectors expressing luciferase and hsps and/or co-chaperones. After 3 days, cells were subjected to heat shock at 46 °C and left for 0–60 minutes before luciferase assays were carried out (Promega, Mannheim, Germany).

Filter-trap assays. Cells were lysed with sodium dodecyl sulfate lysis buffer and centrifuged to obtain insoluble and soluble fractions, as previously described.[17] Samples were applied onto a nitrocellulose or cellulose acetate membrane under vacuum using slot-blot apparatus; proteins within each fraction were detected by probing with various antibodies and visualized using enhanced chemiluminescence reagent.

Western blots. In brief, cells were transduced with virus (at a multiplicity of infection of 100), lysed after 48 hours using radioimmunoprecipitation

assay buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate; Sigma, Dorset, UK) containing protease inhibitors phenyl-methyl-sulphonyl-fluoride (100 μg/ml), aprotinin (70 μg/ml), and sodium orthovanadate (1 mmol/l). Samples were then resolved by sodium dodecyl sulfate gel electrophoresis; proteins were transferred to polyvinylidene fluoride membrane (Roche, Basel, Switzerland) using a wet-transfer method and probed with primary antibodies raised against hsp70i (Stressgen, MI), hsp40 (Stressgen, MI), HSJ1a and HSJ1b [Kindly supplied by Prof. Cheetham[43]] ubiquitin (Ubi1; Abcam), or alpha tubulin (Sigma, Dorset, UK), as indicated.

Co-immunoprecipitation assays. Co-immunoprecipitation was performed using a ProFound co-immunoprecipitation kit (Pierce, Perbio Science, Cheshire, UK). In brief, N2a cells were co-transduced with Ad vectors expressing luciferase and the appropriate control vectors (Ad0) or those expressing HSJ1a. Cells were lysed 40 hours after transduction with co-immunoprecipitation lysis buffer (50 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.9, 250 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4, 20 mmol/l β-glycerophosphate, 5 mmol/l 1,4-dithiothreitol, 0.5% Igepal) containing protease inhibitors (Protease cocktail; Roche, Basel, Switzerland). Cell lysates were centrifuged for 15 minutes at 10,000*g* and the resulting supernatant was incubated with Aminolink plus coupling gel coupled to luciferase antibody (Serotec AbD, Oxford, UK). Samples were boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis loading buffer to elute associated proteins. Western blots were then run, and proteins were transferred onto polyvinylidene fluoride membranes, which were then probed with anti-ubiquitin (1:5,000, Abcam, Cambridge, UK), anti-luciferase (1:1,000; Serotec AbD, Oxford, UK), and HSJ1a (1:1,000) antibodies.

ACKNOWLEDGMENTS

The authors would like to thank the Wellcome Trust, the Biotechnology and Biological Sciences Research Council, Regeneration, and the Motor Neurone Disease Society for supporting and funding this work.

REFERENCES

1. Fu, YH, Kuhl, DP, Pizzuti, A, Pieretti, M, Sutcliffe, JS, Richards, S *et al.* (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* **67**: 1047–1058.
2. La Spada, AR and Taylor, JP (2003). Polyglutamines placed into context. *Neuron* **38**: 681–684.
3. Michalik, A and Van Broeckhoven, C (2003). Pathogenesis of polyglutamine disorders: aggregation revisited. *Hum Mol Genet* **12**: R173–R186.
4. Ordway, JM, Tallaksen-Greene, S, Gutekunst, CA, Bernstein, EM, Cearley, JA, Wiener, HW *et al.* (1997). Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. *Cell* **91**: 753–763.
5. Hodgson, JG, Agopyan, N, Gutekunst, CA, Leavitt, BR, LePiane, F, Singaraja, R *et al.* (1999). A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* **23**: 181–192.
6. Steffan, JS, Kazantsev, A, Spasic-Boskovic, O, Greenwald, M, Zhu, YZ, Gohler, H *et al.* (2000). The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc Natl Acad Sci USA* **97**: 6763–6768.
7. Evert, BO, Wullner, U and Klockgether, T (2000). Cell death in polyglutamine diseases. *Cell Tissue Res* **301**: 189–204.
8. Shimohata, T, Onodera, O and Tsuji, S (2000). Interaction of expanded polyglutamine stretches with nuclear transcription factors leads to aberrant transcriptional regulation in polyglutamine diseases. *Neuropathology* **20**: 326–333.
9. Lipinski, MM and Yuan, J (2004). Mechanisms of cell death in polyglutamine expansion diseases. *Curr Opin Pharmacol* **4**: 85–90.
10. Soto, C (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat Rev Neurosci* **4**: 49–60.
11. Jiang, H, Nucifora, FC Jr., Ross, CA and DeFranco, DB (2003). Cell death triggered by polyglutamine-expanded Huntingtin in a neuronal cell line is associated with degradation of CREB-binding protein. *Hum Mol Genet* **12**: 1–12.
12. Chai, Y, Shao, J, Miller, VM, Williams, A and Paulson, HL (2002). Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis. *Proc Natl Acad Sci USA* **99**: 9310–9315.
13. Arrasate, M, Mitra, S, Schweitzer, ES, Segal, MR and Finkbeiner, S (2004). Inclusion body formation reduces levels of mutant Huntingtin and the risk of neuronal death. *Nature* **431**: 805–810.
14. Dunah, AW, Jeong, H, Griffin, A, Kim, YM, Standaert, DG, Hersch, SM *et al.* (2002). Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. *Science* **296**: 2238–2243.
15. Watase, K, Weeber, EJ, Xu, B, Antalffy, B, Yuva-Paylor, L, Hashimoto, K *et al.* (2002). A long CAG repeat in the mouse Sca1 locus replicates SCA1 features and reveals the impact of protein solubility on selective neurodegeneration. *Neuron* **34**: 905–919.
16. Yu, ZX, Li, SH, Nguyen, HP and Li, XJ (2002). Huntingtin inclusions do not deplete polyglutamine-containing transcription factors in HD mice. *Hum Mol Genet* **11**: 905–914.
17. Bailey, CK, Andriola, IF, Kampinga, HH and Merry, DE (2002). Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. *Hum Mol Genet* **11**: 515–523.
18. Jana, NR, Tanaka, M, Wang, G and Nukina, N (2000). Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Hum Mol Genet* **9**: 2009–2018.
19. Muchowski, PJ, Schaffar, G, Sittler, A, Wanker, EE, Hayer-Hartl, MK and Hartl, FU (2000). Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proc Natl Acad Sci USA* **97**: 7841–7846.
20. Freeman, BC, Myers, MP, Schumacher, R and Morimoto, RI (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO J* **14**: 2281–2292.
21. Minami, Y, Hohfeld, J, Ohtsuka, K and Hartl, FU (1996). Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. *J Biol Chem* **271**: 19617–19624.
22. Morimoto, RI (1993). Cells in stress: transcriptional activation of heat shock genes. *Science* **259**: 1409–1410.
23. Nollen, EA, Kabakov, AE, Brunsting, JF, Kanon, B, Hohfeld, J and Kampinga, HH (2001). Modulation of *in vivo* HSP70 chaperone activity by Hip and Bag-1. *J Biol Chem* **276**: 4677–4682.
24. Garrido, C and Solary, E (2003). A role of HSPs in apoptosis through "protein triage"? *Cell Death Differ* **10**: 619–620.
25. Alberti, S, Demand, J, Esser, C, Emmerich, N, Schild, H and Hohfeld, J (2002). Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. *J Biol Chem* **277**: 45920–45927.
26. Connell, P, Ballinger, CA, Jiang, J, Wu, Y, Thompson, LJ, Hohfeld, J *et al.* (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol* **3**: 93–96.
27. Demand, J, Alberti, S, Patterson, C and Hohfeld, J (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. *Curr Biol* **11**: 1569–1577.
28. Esser, C, Alberti, S and Hohfeld, J (2004). Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochim Biophys Acta* **1695**: 171–188.
29. Jiang, J, Ballinger, CA, Wu, Y, Dai, Q, Cyr, DM, Hohfeld, J and Patterson, C (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *J Biol Chem* **276**: 42938–42944.
30. Luders, J, Demand, J and Hohfeld, J (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *J Biol Chem* **275**: 4613–4617.
31. Chapple, JP, van der Spuy, J, Poopalasundaram, S and Cheetham, ME (2004). Neuronal DnaJ proteins HSJ1a and HSJ1b: a role in linking the Hsp70 chaperone machine to the ubiquitin-proteasome system? *Biochem Soc Trans* **32**: 640–642.
32. Jana, NR, Dikshit, P, Goswami, A, Kotliarova, S, Murata, S, Tanaka, K *et al.* (2005). Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. *J Biol Chem* **280**: 11635–11640.
33. Jana, NR and Nukina, N (2005). BAG-1 associates with the polyglutamine-expanded huntingtin aggregates. *Neurosci Lett* **378**: 171–175.
34. Westhoff, B, Chapple, JP, van der Spuy, J, Hohfeld, J and Cheetham, ME (2005). HSJ1 is a neuronal shuttling factor for the sorting of chaperone clients to the proteasome. *Curr Biol* **15**: 1058–1064.
35. Cheetham, ME, Jackson, AP and Anderton, BH (1994). Regulation of 70-kDa heat-shock-protein ATPase activity and substrate binding by human DnaJ-like proteins, HSJ1a and HSJ1b. *Eur J Biochem* **226**: 99–107.
36. Butler, R, Leigh, PN, McPhaul, MJ and Gallo, JM (1998). Truncated forms of the androgen receptor are associated with polyglutamine expansion in X-linked spinal and bulbar muscular atrophy. *Hum Mol Genet* **7**: 121–127.
37. Darrington, RS, Butler, R, Leigh, PN, McPhaul, MJ and Gallo, JM (2002). Ligand-dependent aggregation of polyglutamine-expanded androgen receptor in neuronal cells. *Neuroreport* **13**: 2117–2120.
38. Alberti, S, Esser, C and Hohfeld, J (2003). BAG-1—a nucleotide exchange factor of Hsc70 with multiple cellular functions. *Cell Stress Chaperones* **8**: 225–231.
39. Graham, FL and Prevec, L (1995). Methods for construction of adenovirus vectors. *Mol Biotechnol* **3**: 207–220.
40. Harding, TC, Geddes, BJ, Murphy, D, Knight, D and Uney, JB (1998). Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system. *Nat Biotechnol* **16**: 553–555.
41. Wong, LF, Ralph, GS, Walmsley, LE, Bienemann, AS, Parham, S, Kingsman, SM *et al.* (2005). Lentiviral-mediated delivery of Bcl-2 or GDNF protects against excitotoxicity in the rat hippocampus. *Mol Ther* **11**: 89–95.
42. Paxinos, G and Watson, C (1986). *The Rat Brain in Stereotaxic Coordinates*, 2nd edn., Academic Press: New York.
43. Chapple, JP and Cheetham, ME (2003). The chaperone environment at the cytoplasmic face of the endoplasmic reticulum can modulate rhodopsin processing and inclusion formation. *J Biol Chem* **278**: 19087–19094.