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Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease

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Huntington's disease (HD) is a progressive neurodegenerative disorder with no effective treatment. Geldanamycin is a benzoquinone ansamycin that binds to the heat shock protein Hsp90 and activates a heat shock response in mammalian cells. In this study, we show by using a filter retardation assay and immunofluorescence microscopy that treatment of mammalian cells with geldanamycin at nanomolar concentrations induces the expression of Hsp40, Hsp70 and Hsp90 and inhibits HD exon 1 protein aggregation in a dose-dependent manner. Similar results were obtained by overexpression of Hsp70 and Hsp40 in a separate cell culture model of HD. This is the first demonstration that huntingtin protein aggregation in cells can be suppressed by chemical compounds activating a specific heat shock response. These findings may provide the basis for the development of a novel pharmacotherapy for HD and related glutamine repeat disorders.

INTRODUCTION

Although since the cloning of the Huntington's disease (HD) gene significant advances have been made in the understanding of the molecular mechanisms underlying this neurodegenerative disease, there is still no effective treatment for HD. HD is caused by an unstable CAG trinucleotide repeat expansion located in exon 1 of the *IT-15* gene encoding huntingtin, a ~350 kDa protein of unknown function (1–3). Evidence has been presented that the formation of neuronal inclusions with aggregated huntingtin protein is associated with the progressive neuropathology in HD (4). However, it is unclear today whether the process of aggregate formation is the cause of HD or merely a consequence of this disorder (5–7). Using *in vitro* model systems we have demonstrated recently that the formation of huntingtin protein aggregates critically depends on polyglutamine repeat length, protein concentration and time (8,9). Furthermore, formation of insoluble aggregates with a fibrillar amyloid-like morphology can be inhibited by small chemical compounds such as Congo red and thioflavine S and the monoclonal antibody 1C2 that specifically recognizes an elongated polyglutamine tract (10). This suggested that inhibition of huntingtin protein aggregation in patients by small molecules could be a promising therapeutic strategy.

Histochemical studies revealed that inclusions containing insoluble polyglutamine-containing protein aggregates in brains of patients and transgenic animals are immunoreactive for ubiquitin, various molecular chaperones and components of the 20 S proteasome (2,11). This suggests that neuronal cells recognize the aggregated huntingtin protein as abnormally folded and, by recruiting chaperones and proteasomal components, try to disaggregate and/or degrade the mutant protein. Consistent with this view, overexpression of the heat shock proteins Hsp40, Hsp70 and Hsp104 in cell culture, yeast, *Caenorhabditis elegans* and fly model systems has blocked the accumulation of polyglutamine-containing protein aggregates (12–15). However, whether the formation of insoluble protein aggregates can be suppressed by activation of a heat shock response is unknown.

Geldanamycin (GA) is a naturally occuring antitumor drug that has been shown to be active in tumor cell lines as well as in mouse models (16). The antitumor effects of GA result from its ability to deplete cells from proto-oncogenic protein kinases and nuclear hormone receptors (17–19). Initially it was thought that GA is a non-specific protein kinase inhibitor. However, subsequent biochemical and structural studies have demonstrated that GA binds specifically to the heat shock protein Hsp90, thereby inhibiting its chaperone function (20–22). Hsp90 is specifically involved in folding and conformational regulation of several medically relevant signal transduction molecules, including nuclear receptors and proto-oncogenic kinases (18,23). Inhibition of Hsp90 function by GA causes degradation of these regulatory proteins (18,24). Recently, Zou *et al.* (25) have shown that GA also disrupts a complex consisting of Hsp90 and the heat shock transcription factor HSF1 and triggers the activation of a heat shock response in mammalian cells. Thus, treatment of cells with GA should induce the expression of the molecular chaperones Hsp70 and Hsp40, which have been shown to be effective in suppressing protein aggregation in model systems of HD and related glutamine repeat disorders (14,15,26).

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In this study we have examined the effect of GA on the formation of insoluble huntingtin exon 1 aggregates in a cell culture model of HD. We found that treatment of cells with GA leads to enhanced expression of Hsp40, Hsp70 and Hsp90 and to inhibition of huntingtin exon 1 protein aggregation. Similar results were obtained by co-expressing Hsp70 and Hsp40 with HD exon 1 protein containing a polyglutamine tract in the pathological range (51 glutamines). Models that explain the effect of GA treatment on huntingtin protein aggregation are discussed.

RESULTS

GA activates a heat shock response in mammalian cells

In order to induce a heat shock response, COS-1 cells expressing the fusion of enhanced green fluorescent protein (EGFP) and the huntingtin exon 1 protein with 72 glutamines (HD72Q) were treated with various concentrations of GA. Forty hours post-transfection, total cell extracts were prepared and expression of EGFP-HD72Q and the heat shock proteins Hsp40, Hsp70 and Hsp90 was examined by immunoblot analysis using specific antibodies. As shown in Figure 1A, soluble EGFP-HD72Q protein migrating in the SDS-gel at ~57 kDa was detected in protein extracts prepared from transfected cells (lanes 1–6) but not in protein extracts of untransfected control cells (lane 7). Treatment of cells with increasing concentrations of GA (18–360 nM) had no effect on the production of soluble EGFP-HD72Q protein. In contrast, the expression of each of the molecular chaperones Hsp40, Hsp70 and Hsp90 increased with increasing GA concentrations (lanes 1–4), indicating that treatment of cells with GA triggers a heat shock response. Addition of GA to a final concentration of 360 nM resulted in a 3- to 4-fold up-regulation of Hsp40, Hsp70 and Hsp90 compared with the untreated controls.

Activation of a heat shock response by GA inhibits huntingtin protein aggregation

To determine whether induction of Hsp40, Hsp70 and Hsp90 expression by GA treatment has an effect on EGFP-HD72Q aggregation, COS-1 cells grown in the presence of various concentrations of GA were lysed and analyzed by a filter retardation assay for the presence of aggregated huntingtin protein (27). Using this assay, SDS-resistant HD exon 1 protein aggregates can be immunologically detected and quantified. As shown in Figure 1B, treatment of cells with GA resulted in a concentration-dependent inhibition of SDS-insoluble EGFP-HD72Q protein aggregates. At 18, 90, 180 and 360 nM, GA reduced the amount of insoluble protein aggregates by ~30, 60, 70 and 80%, respectively, as detected by the filtration assay.

The results obtained by the filter retardation assay were confirmed by fluorescence microscopy. Whereas in untreated control cells (Fig. 2A and B) large perinuclear EGFP-HD72Q protein aggregates with a diameter of 2–5 μm were detected, these structures were no longer visible in GA-treated cells (Fig. 2C–F). At GA concentrations of 18–90 nM the large perinuclear inclusion bodies were replaced by smaller dot-like protein aggregates (diameter 0.1–0.5 μm) that were dispersed throughout the cytoplasm. At higher GA concentrations (180–360 nM) these smaller aggregates were no longer detectable, indicating that GA is a potent inhibitor of HD exon 1 protein aggregation in mammalian cells.

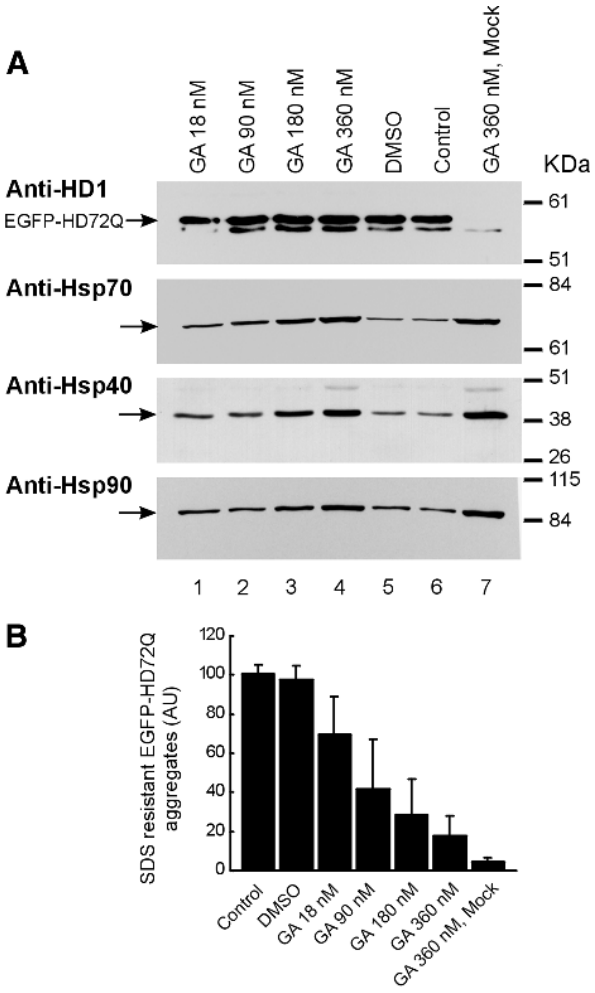

Figure 1. GA induces a heat shock response and inhibits aggregation of EGFP-HD72Q in COS-1 cells. (**A**) Expression of EGFP-HD72Q, Hsp40, Hsp70 and Hsp90 in COS-1 cells. Cells expressing EGFP-HD72Q were treated for 40 h with increasing concentrations of GA. Protein extracts prepared from GA-treated and untreated cells (control) were analyzed by SDS–PAGE and immunoblotting using the indicated antibodies. Equal amounts (10 μg) of protein were loaded. (**B**) GA treatment of COS-1 cells prevents the formation of SDS-insoluble EGFP-HD72Q protein aggregates. Aggregates were detected using the filter retardation assay. Filters were probed with the HD1 antibody and signal intensities quantified using a Fuji-imager (LAS 2000). The signal intensity obtained from the sample without added GA was arbitrarily set as 100 (control). Values shown are the mean of three independent experiments (± standard error).

Hsp40 and Hsp70 co-localize with mutant HD exon 1 in GA-treated cells

To examine whether the molecular chaperones Hsp40, Hsp70 and Hsp90 co-localize with mutant huntingtin protein, GA-treated COS-1 cells were permeabilized and analyzed by indirect immunofluorescence microscopy. Comparison of the fluorescence of EGFP-HD72Q with the immunostaining of Hsp40 and Hsp70 revealed that both chaperones co-localize with the mutant huntingtin protein (Fig. 3A–F). At a GA concentration of 360 nM, EGFP-HD72Q as well as the chaperones Hsp40

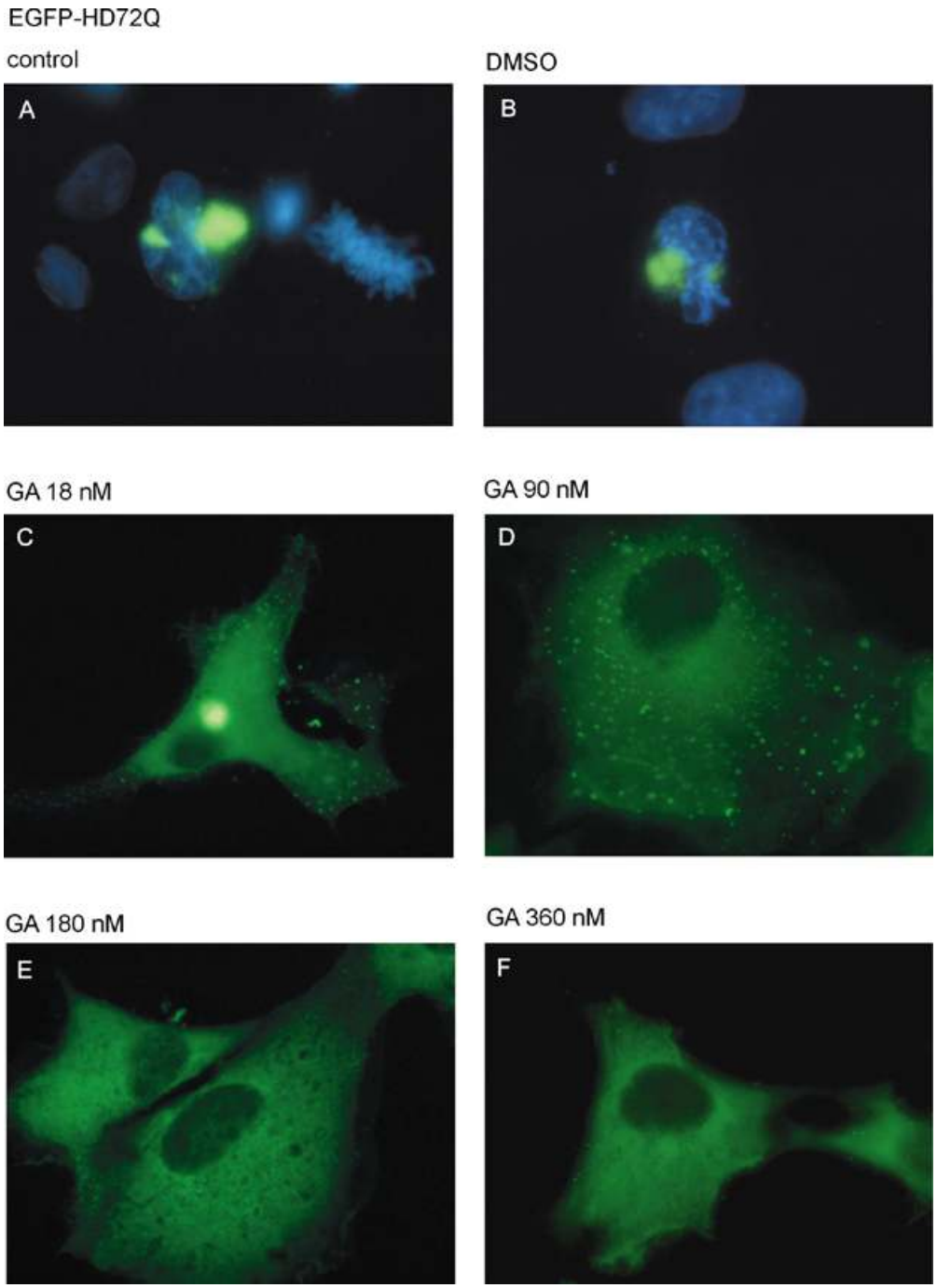

Figure 2. Fluorescence analysis of GA-treated COS-1 cells expressing EGFPHD72Q. COS-1 cells grown for 24 h in the absence (**A** and **B**) or presence of GA (**C**–**F**) were examined for EGFP-HD72Q expression by fluorescence microscopy (green). Nuclei were counterstained with Hoechst.

and Hsp70 were evenly distributed in the cytoplasm and no perinuclear inclusion bodies with aggregated huntingtin protein were observed. Interestingly, under the same conditions, fluorescence of EGFP-HD72Q only partially overlapped with the immunostaining of Hsp90 (Fig. 3G–I), suggesting that a physical interaction of Hsp90 with the aggregation-prone HD exon 1 protein is not required to prevent aggregate formation. A direct interaction of Hsp40 and Hsp70 with EGFP-HD72Q, however, appears to be critical for inhibition of polyglutamine assembly, consistent with previous findings (26).

Overexpression of Hsp70 and Hsp40 inhibits HD exon 1 protein aggregation in COS-1 cells

To determine whether overexpression of heat shock proteins mimics the GA effect on HD exon 1 protein aggregation, the Flag- and HA-tagged heat shock proteins Hdj1 (Hsp40) and

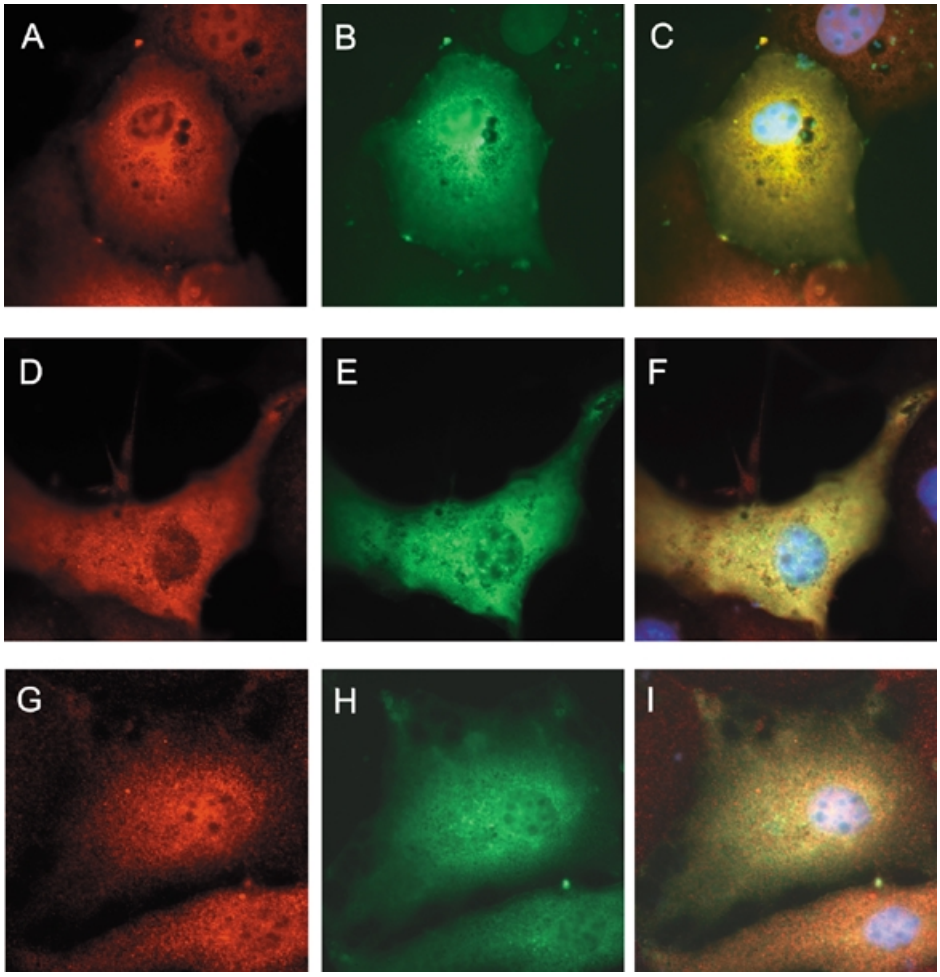

Figure 3. Co-localization of EGFP-HD72Q with Hsp40, Hsp70 and Hsp90 in GA-treated COS-1 cells. Following incubation of cells for 40 h with GA at 360 nM, cells expressing EGFP-HD72Q (green) were immunolabeled with antibodies directed against Hsp40 (**A**–**C**), Hsp70 (**D**–**F**) and Hsp90 (**G**–**I**) coupled to a Cy3-conjugated secondary antibody (red). Co-localization of EGFP-HD72Q with Hsp40, Hsp70 and Hsp90 is shown in (C), (F) and (I), respectively. Nuclei were counterstained with Hoechst.

Hsp70, respectively, were transiently co-expressed with mutant HD51Q protein in COS-1 cells. Protein extracts were prepared 40 h post-transfection and analyzed by SDS–PAGE and immunoblotting. As shown in Figure 4A, the recombinant proteins HD51Q, Flag-Hdj1 and HA-Hsp70, migrating in the SDS-gel at ~30, 40 and 73 kDa, respectively, were detected in transfected but not in untransfected cells. In transfected cells both HA-Hsp70 and Flag-Hdj1 chaperones were over-expressed ~4-fold compared with the endogenous levels (data not shown). The effect of chaperone overexpression on HD51Q aggregation is shown in Figure 4B. Co-expression of either Flag-Hdj1 or HA-Hsp70 with HD51Q resulted in an ~30–40% reduction of the amount of SDS-insoluble HD exon 1 aggregates in COS-1 cells. In comparison, when both chaperones were simultaneously co-expressed with HD51Q the amount of insoluble aggregates formed was diminished by 60–80%, indicating that a cooperation between Flag-Hdj1 and HA-Hsp70 is required for an efficient inhibition of HD51Q aggregation in COS-1 cells. Co-expression of Hsp90 with HD51Q had no discernible effect on the formation of insoluble protein aggregates, suggesting that this chaperone is not directly involved in the inhibition of HD exon 1 protein aggregation in mammalian cells (data not shown).

Analysis by indirect immunofluorescence microscopy revealed that neither the overexpression of Flag-Hjd1 nor that of HA-Hsp70 was able to prevent the accumulation of large perinuclear inclusions with aggregated HD51Q protein (Fig. 5A–F). In strong contrast, when both chaperones were co-expressed with HD51Q the large perinuclear inclusion bodies totally disappeared and smaller dot-like aggregates with a diameter of 0.2–0.5 μm were observed (Fig. 5G–I). These aggregates were dispersed throughout the cytoplasm and were structurally similar to the ones observed after treatment of COS-1 cells with lower concentrations (18–90 nM) of GA (Fig. 2C and D).

Overexpression of Hsp70 and Hsp40 prevents formation of fibrillar protein aggregates

As morphological changes of protein aggregates in cells are poorly detectable by immunofluorescence microscopy, we also examined the effect of chaperone overexpression on aggregate formation by electron microscopy. At the ultrastructural level,

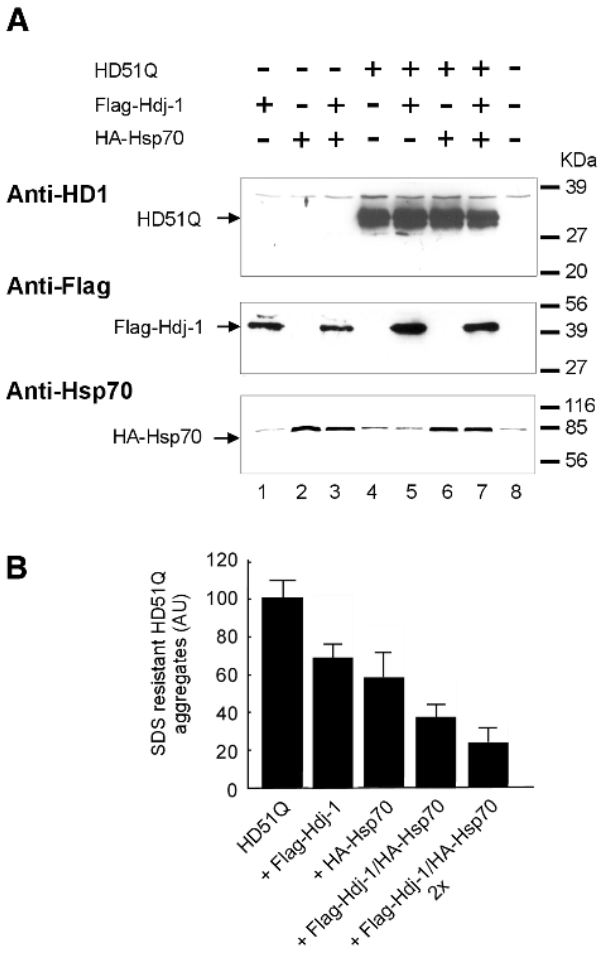

Figure 4. Overexpression of Flag-Hdj-1 and HA-Hsp70 inhibits HD51Q protein aggregation in COS-1 cells. (**A**) Western blot analysis. COS-1 cells were transfected with constructs as indicated at the top of the figure. Forty hours post-transfection, protein extracts were prepared and analyzed by SDS–PAGE and immunoblotting using specific antibodies. The anti-Hsp70 antibody detects both transiently expressed HA-Hsp70 and endogenous Hsp70. Equal amounts (10 μg) of protein were loaded. (**B**) Inhibition of HD51Q aggregation by overexpression of Flag-Hdj1 and HA-Hsp70. Aggregates were detected and quantified as in Figure 1B. The signal intensity obtained from the sample without overexpression of heat shock proteins (HD51Q) was arbitrarily set as 100. Data represent means of five independent experiments (± standard error). 2×, double the amount of plasmid DNA was transfected.

most cells expressing HD51Q contained large perinuclear inclusion bodies (diameter 1–5 μm) composed of electron-dense filamentous material (Fig. 6A–C). The identity of the HD51Q fibrils was confirmed by immunoelectron microscopy using the anti-huntingtin antibodies AG51 (Fig. 6A and B) or HD1 (not shown) and a gold colloid secondary antibody. Interestingly, the anti-AG51 antibody immunolabeled mainly the periphery but not the interior of the inclusion bodies, suggesting that the HD exon 1 protein in the inclusion bodies is so densely packed that it is no longer accessible for the antibodies. Both Flag-Hdj1 and HA-Hsp70 co-localized with the perinuclear inclusion bodies; however, their association did not significantly alter the fibrillar structure of the HD51Q protein aggregates (Fig. 6D and E). As expected, in cells co-expressing Flag-Hdj1 and HA-Hsp70 no perinuclear HD51Q inclusion bodies were detected, once again indicating that overexpression of both heat shock proteins suppresses aggregate formation. Although more than 200 different cells co-expressing Flag-Hdj1/HA-Hsp70/HD51Q were examined by immunoelectron microscopy, in none of these cells could large inclusion bodies with aggregated HD51Q protein be observed. The mutant HD51Q protein appeared to be distributed homogenously in the cytoplasm of the transfected cells (Fig. 6F).

DISCUSSION

In this study we show for the first time that treatment of mammalian cells with the ansamycin antibiotic GA results in the activation of a heat shock response which subsequently prevents the self-assembly of HD exon 1 protein with a polyglutamine sequence in the pathological range (51 and 72 glutamines). Our results further demonstrate that a similar effect is obtained when both Hsp40 and Hsp70 are overexpressed in cells, indicating that a synergistic action of these two heat shock proteins is critical for inhibition of protein aggregation *in vivo*. However, additional studies will be necessary to address the sequence of events involved in this process, starting with the induction of the heat shock response by GA treatment and leading eventually to inhibition of aggregate formation. GA has been shown to inhibit ATP binding to Hsp90 because it occupies the ATP binding site with high affinity (21,22). Thus, any cellular effects of GA must be interpreted as a consequence of the interruption of the Hsp90 ATPase cycle. Treatment of cells with GA blocks the maturation of Hsp90 protein substrates and the refolding of certain stress-denatured polypeptides, which results in the efficient degradation of these substrates by the proteasome system (24). This effect is the basis for the potential therapeutic application of GA and its derivatives. It has been suggested that it can be used for depleting the cellular levels of steroid receptors and Hsp90-dependent proto-oncogenic protein kinases (18,19).

In other studies evidence has been presented that treatment of cells with GA results in the dissociation of Hsp90-containing multichaperone complexes. Zou *et al.* (25) have demonstrated that GA activates the heat shock transcription factor HSF1 which results in the accumulation and trimerization of unbound HSF1 in the cytoplasm of mammalian cells. This complex then translocates from the cytoplasm to the nucleus where it becomes trancriptionally competent (28). In the nucleus HSF1 specifically binds to the heat shock element sequences present in *hsp* promoters and activates the expression of several molecular chaperones. We propose that the effect of GA on HD exon 1 aggregation is a direct consequence of the activation of Hsp40, Hsp70 and Hsp90 expression. Once these proteins are overproduced they associate with the misfolded mutant HD exon 1 protein and prevent its aggregation into fibrillar structures. Our results indicate that a cooperation between Hsp40 and Hsp70 is critical for the inhibition of mutant HD exon 1 aggregation in mammalian cells, whereas Hsp90 itself does not seem to be directly involved in this process. Our data are consistent with previous studies on huntingtin aggregation (26) and are also supported by the finding that both Hsp40 and Hsp70 co-localize with mutant EGFP-HD72Q in GA-treated cells, whereas Hsp90 does not or only partially co-localizes with the mutant HD exon 1 protein.

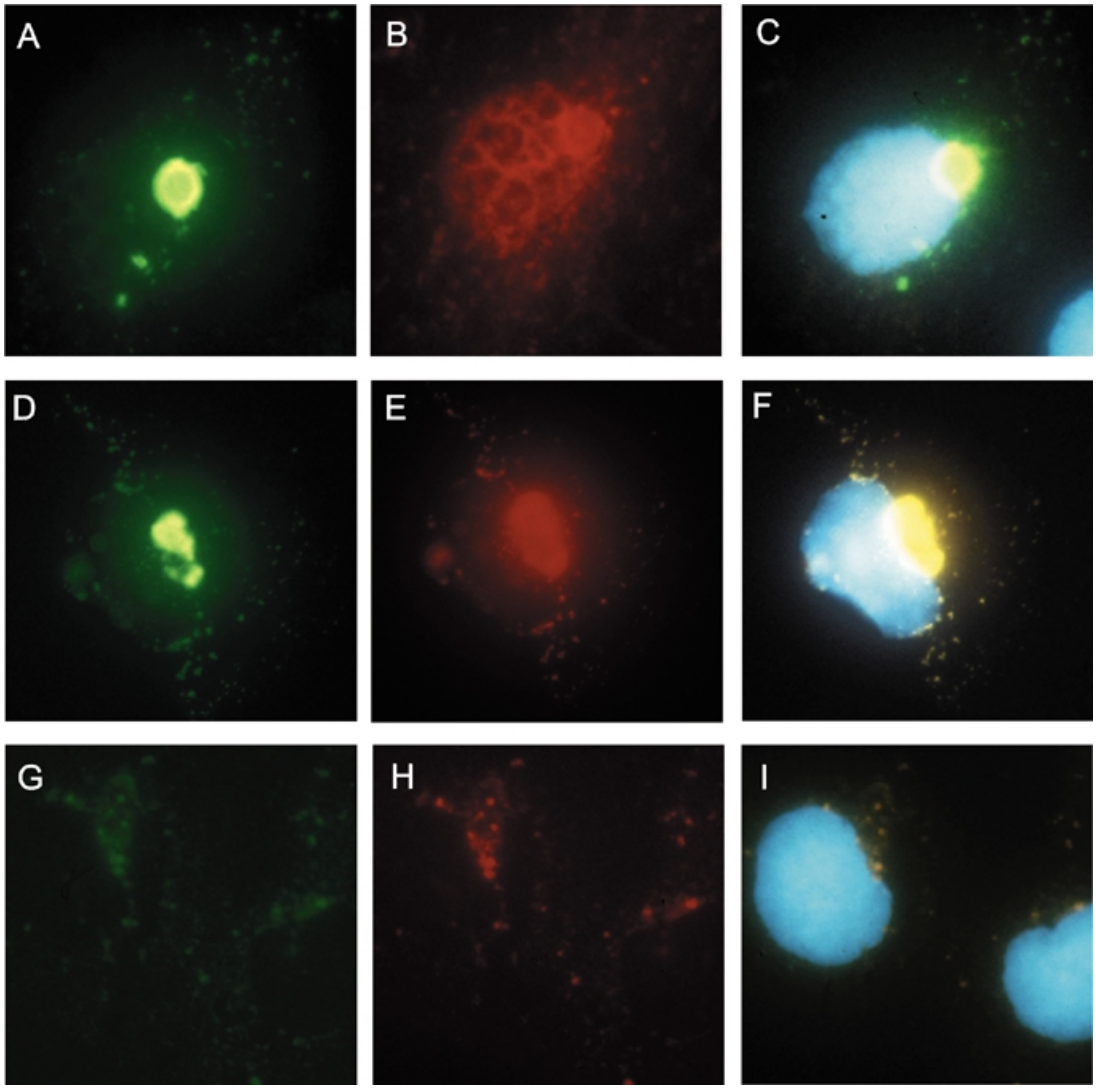

Figure 5. Immunofluorescence analysis of HD51Q aggregation in COS-1 cells. Forty-two hours post-transfection, COS-1 cells co-expressing HD51Q/Flag-Hdj1 (**A**–**C**), HD51Q/HA-Hsp70 (**D**–**F**) or HD51Q/Flag-Hdj1/HA-Hsp70 (**G**–**I**) were examined by indirect immunofluorescence microscopy. HD51Q protein aggregates were immunolabeled with the HD1 antibody coupled to a FITC-conjugated secondary antibody (green). Flag-Hdj1 and HA-Hsp70 were labeled with anti-Flag and anti-Hsp70 antibodies, respectively, coupled to a Cy3-conjugated secondary antibody (red). Nuclei were counterstained with Hoechst.

In addition, co-expression of epitope-tagged Hsp40 and Hsp70 efficiently blocked the formation of HD51Q protein aggregates whereas overexpression of Hsp90 had no effect. Thus, we suggest that a direct physical interaction between Hsp90 and HD exon 1 protein is not required for suppression of aggregate formation *in vivo*. Rather it appears that the key trigger is the GA-induced dissociation of HSF-Hsp90 complexes. This leads to activation of the expression of Hsp40 and Hsp70 which then inhibit the accumulation of mutant HD exon 1 in mammalian cells.

Recently, Chan *et al.* (14) have demonstrated that over-expression of Hsp70 and Hsp40 suppressed polyglutamine-induced neurodegeneration and toxicity in a *Drosophila melanogaster* model system for Machado–Joseph disease (MJD/SCA3). Together with our data on HD, this suggests that stimulation of chaperone expression might be used to modulate polyglutamine-induced pathogenesis in patients. We propose that the activation of a heat shock response in neuronal cells of patients by chemical compounds could be a reasonable therapeutic strategy for HD. Interestingly, GA is currently being tested as an antitumor drug in clinical trials. However, it is well known that GA, even at low concentrations, is toxic for cells, which might limit its suitability for the treatment of patients over longer periods of time (16). More extensive research into the tolerability of GA are currently under way and perhaps derivatives, such as 17-allylamino,17-demethoxy-geldanamycin or radicicol can be further developed into a therapeutic drug against polyglutamine disorders (29,30). Importantly, such drugs should cross the blood–brain barrier to a significant degree.

Although it has not been formally proven that the deposition of huntingtin protein aggregates in neuronal cells (31) is the cause of HD, we propose that finding chemical compounds that directly prevent aggregate formation or induce a heat shock response will significantly delay the onset as well as the progression of polyglutamine repeat disorders such as HD.

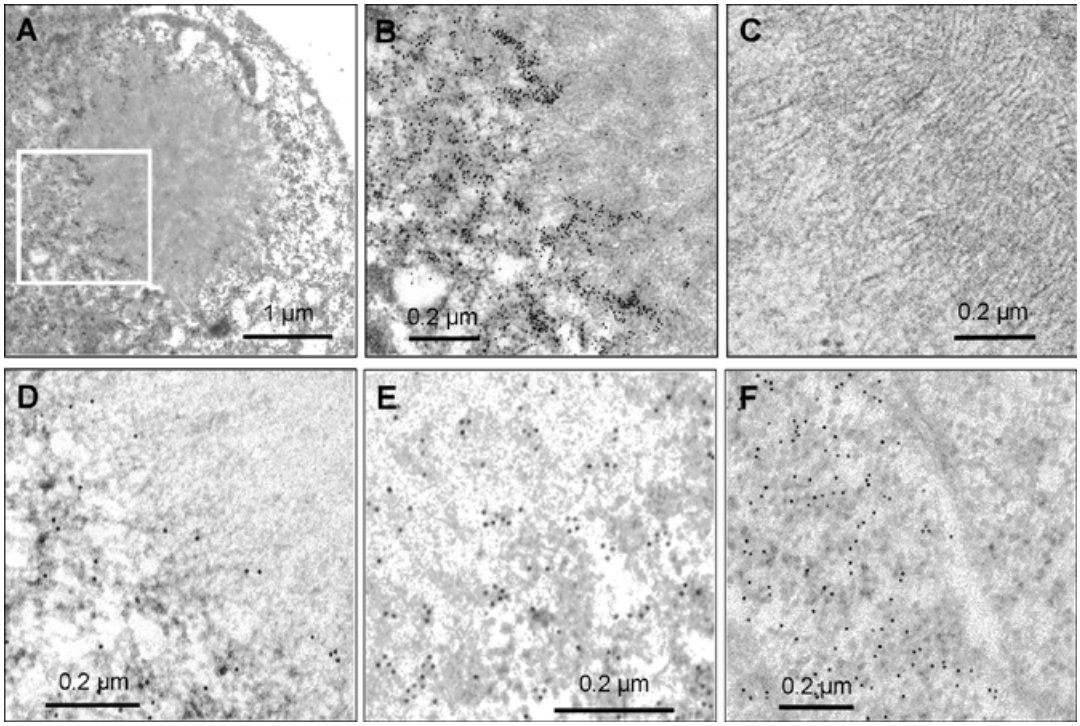

Figure 6. Ultrastructural analysis of HD51Q aggregates following Flag-Hdj1 and HA-Hsp70 overexpression. COS-1 cells expressing HD51Q alone (**A–C**) or co-expressing HD51Q/Flag-Hdj1 (**D**), HD51Q/HA-Hsp70 (**E**) or HD51Q/Flag-Hdj1/HA-Hsp70 (**F**) were viewed by electron microscopy. (A–C) Different magnifications of a cell containing a typical perinuclear inclusion body. At higher magnification HD51Q fibrils can be observed (C). Immunogold labeling of cells with the anti-AG51 antibody confirms the identity of the HD51Q fibrils (B). Immunogold labeling of cells also reveals that Flag-Hdj1 (D) and HA-Hsp70 (E) are associated with HD51Q fibrils. In cells co-expressing HD51Q/Flag-Hdj1/HA-Hsp70 no HD51Q fibrils but homogenous cytoplasmic labeling was observed with the HD1 antibody (F).

Furthermore, such molecules will help to elucidate the key steps in the pathogenesis of HD.

MATERIALS AND METHODS

Plasmid constructions

Exon 1 of the human HD gene containing 51 glutamines was derived from pCAG51 (32) and cloned into pTL1 (33), resulting in construct pTL1-CAG51. pTL1-HA was generated by insertion of a Kozak sequence (34) and a sequence encoding an HA-tag (MAYPYDVPDYASLRS) into pTL1. A further linker was introduced in order to generate the appropriate reading frame, resulting in pTL1-HA3. Hsp70-pTLHA3 was generated by PCR amplification of the human Hsp70A gene and cloning into pTL1-HA3. Hdj-1-pTL10Flag was generated by ligating the human HDJ-1 gene, derived from pQE9-His-Hsp40 (35), into pTL10SFlag (a kind gift from D. Devys and J.-L. Mandel). pEGFP-HD72Q was generated by PCR amplification of the HD exon 1 from patient DNA and cloning into pEGFP-C1 (Clontech). All constructs were verified by sequencing.

Antibodies

The following antibodies were used for western blot (WB) and/or immunofluorescence analysis (IF): rabbit polyclonal HD1 IgG (32) diluted 1:5000 (WB) or 1:1000 (IF), rabbit polyclonal AG51 IgG (8) diluted 1:100 (immunolabeling in electron microscopy), goat polyclonal anti-Hsp70 (Santa Cruz Biotechnology) diluted 1:2000 (WB) or 1:200 (IF), mouse monoclonal anti-Hsp70 (Santa Cruz Biotechnology) diluted 1:5000 (WB), rabbit polyclonal anti-Hsp40 (StressGen) diluted 1:10 000 (WB) or 1:500 (IF), rabbit polyclonal anti-Hsp90 (Santa Cruz Biotechnology) diluted 1:1000 (WB) or 1:100 (IF), mouse monoclonal anti-HA (Boehringer Mannheim) diluted 1:2000 (WB) or 1:200 (IF), and mouse monoclonal M2 anti-Flag (Sigma) diluted 1:10 000 (WB) or 1:1000 (IF).

Cell lines and cell transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 5% fetal calf serum and containing penicillin (100 U/ml) and streptomycin (100 µg/ml). Transfection was performed by the calcium phosphate co-precipitation technique (36). For the expression of the HD51Q, Flag-Hdj-1 and HA-Hsp70 proteins, cells were plated to 30% confluence in 90 mm plates and co-transfected with 3 µg of pTL1-CAG51, 3 or 6 µg of Hsp70-pTLHA3 and 3 or 6 µg of Hdj-1-pTL10Flag along with 5–11 µg of carrier pBluescript DNA. After 16 h the calcium phosphate precipitate was washed from the cells and new medium was added to the plates. Forty to 42 h after transfection the cells were harvested and lysed in the presence of protease inhibitors.

GA (GibcoBRL Life Technologies), at 1.8 mM stock in DMSO was diluted into fresh medium to give final concentrations of 18–360 nM and added to cells at the time of transfection. After 16 h cells were washed and new medium containing GA was added. A further medium change with GA was done 24 h after transfection. Control cells were treated with DMSO. As an alternative transfection method, the Lipofectamine Plus reagent (GibcoBRL Life Technologies) was used according to the manufacturer's instruction.

Preparation of protein extracts

Cell lysis and preparation of the soluble and insoluble protein fractions were performed as described by Sittler *et al.* (37). For preparation of whole cell extracts, cell lysis was performed on ice for 30 min in buffer containing protease inhibitors, and nucleic acids were digested with 125 U/ml Benzonase (Merck). Protein concentration was determined by the BioRad assay.

Western blot analysis and filter retardation assay

SDS–PAGE and western blot analysis were performed according to standard procedures. For the filter retardation assay (27,32) protein samples (1–20 µg) were heated at 98°C for 3 min in 2% SDS and 50 mM DTT and filtered through a 0.2 µm cellulose acetate membrane (Schleicher & Schuell) using a BRL dot-blot filtration unit. Captured aggregates were detected by incubation with HD1 IgG (diluted 1:5000) followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG and the fluorescent substrate AttoPhos. Quantitation of the captured aggregates was performed using a Fuji-Imager (LAS 2000) and AIDA 1.0 image analysis software.

Immunofluorescence and electron microscopy

Immunofluorescence microscopy of transfected COS-1 was performed as described by Sittler *et al.* (37) using the anti-huntingtin HD1 IgG (1:1000) coupled to FITC-conjugated donkey anti-rabbit IgG (1:200, Jackson Immuno Research Laboratories), the mouse monoclonal anti-FLAG antibody (1:1000, Sigma) coupled to Cy3-conjugated donkey anti-mouse IgG (1:200, Jackson Immuno Research Laboratories), the goat polyclonal anti-Hsp70 antibody (1:200, Santa Cruz Biotechnology) coupled to Cy3-conjugated donkey anti-goat IgG (1:200, Jackson Immuno Research Laboratories), the anti-Hsp40 (1:500, StressGen) and the anti-Hsp90 (1:300, StressGen) coupled to Cy3-conjugated secondary antibodies. Nuclei were counterstained with Hoechst (bis-benzimide, Sigma). The samples were examined with a fluorescence microscope Axioplan-2 (Zeiss). COS-1 cells transfected with pEGFP-HD72Q were fixed with 2% paraformaldehyde for 4 min at room temperature followed by direct observation of the green fluorescent fusion protein.

For electron microscopic analysis, monolayers of cells were fixed with 1% formaldehyde, 0.2% glutaraldehyde for 1 h, dehydrated in an ethanol series and embedded in LR Gold (London Resin Company). Post-embedding immunogold labeling was performed as described by Waelter *et al.* (38) using the anti-huntingtin antibodies HD1 (1:400) and AG51 (1:100), or goat anti-Hsp70 (1:400) and goat anti-Hsp40 (1:150) antibodies, followed by secondary antibodies conjugated with 10 nm gold (1:100, British Bio Cell). Sections were poststained with uranyl acetate and lead citrate. Samples were viewed in a Philips CM100 electron microscope.

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