Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy

Brinda Ravikumar[1], Rainer Duden[2] and David C. Rubinsztein[1],*

[1]Department of Medical Genetics and [2]Department of Clinical Biochemistry, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK

Protein conformational disorders (PCDs), such as Alzheimer's disease, Huntington's disease (HD), Parkinson's disease and oculopharyngeal muscular dystrophy, are associated with proteins that misfold and aggregate. Here we have used exon 1 of the HD gene with expanded polyglutamine [poly(Q)] repeats and enhanced green fluorescent protein tagged to 19 alanines as models for aggregate-prone proteins, to investigate the pathways mediating their degradation. Autophagy is involved in the degradation of these model proteins, since they accumulated when cells were treated with different inhibitors acting at distinct stages of the autophagy–lysosome pathway, in two different cell lines. Furthermore, rapamycin, which stimulates autophagy, enhanced the clearance of our aggregate-prone proteins. Rapamycin also reduced the appearance of aggregates and the cell death associated with the poly(Q) and polyalanine [poly(A)] expansions. Since rapamycin is used clinically, this drug or related analogues may be suitable candidates for therapeutic investigation in HD and related diseases. We have also re-examined the role of the proteasome, since previous studies in poly(Q) diseases have used lactacystin as an inhibitor — recent studies have shown that lactacystin may also affect lysosomal function. Both lactacystin and the specific proteasomal inhibitor epoxomicin increased soluble protein levels of the poly(Q) constructs, suggesting that these are also cleared by the proteasome. However, while poly(Q) aggregation was enhanced by lactacystin in our inducible PC12 cell model, aggregation was reduced by epoxomicin, suggesting that some other protein(s) induced by epoxomicin may regulate poly(Q) aggregation.

INTRODUCTION

Proteinopathies or protein conformational disorders (PCDs), such as Alzheimer's disease, Huntington's disease (HD), Parkinson's disease and oculopharyngeal muscular dystrophy (OPMD), are associated with particular proteins or sets of proteins that misfold and aggregate in specific tissues (1). However, it is controversial and unclear whether the aggregates (also known as inclusions) seen in these diseases or the monomeric proteins are directly toxic, or whether toxicity is mediated by the aggregation process itself.

Some PCDs are caused by codon reiteration mutations, where protein misfolding is mediated by the abnormal expansion of a tract of repeated amino acids. These include polyglutamine [poly(Q)] expansion diseases, exemplified by HD. HD is characterized by expansions of a poly(Q) stretch in exon 1 of the Huntingtin gene to more than 37 glutamines, and a short N-terminal fragment encoding the polyglutamine stretch is sufficient to cause aggregates in mice (2,3) and in cell models (4–6). Indeed, many believe that the mutant protein acquires its toxicity and its propensity to aggregate after cleavage, forming a short (so far incompletely defined)

N-terminal fragment containing the polyglutamine stretch (5). More recently, polyalanine [poly(A)] expansion mutations in the polyadenine-binding protein 2 gene have been shown to cause OPMD, which is associated with aggregates in muscle cell nuclei (7). This disease has been modelled in cell culture systems, where aggregate formation is associated with cell death (8). We have recently shown that polyA expansions of 19 or more repeats tagged with enhanced green fluorescent protein are sufficient to cause intracytoplasmic aggregate formation and cell death in cultured cells (9).

Many of the codon reiteration diseases (and other PCDs) are dominantly inherited, and genetic and transgenic studies suggest that they are generally due to gain-of-function mutations [e.g. in poly(Q) diseases] (reviewed in 10). In many cases, disease severity correlates with the expression levels of the mutant protein (reviewed in 10). Thus, it is important to understand the factors regulating clearance of these aggregate-prone proteins and of the aggregates themselves. The traditional view is that such proteins are expected to be cleared by the ubiquitin–proteasome pathway, where malfolded proteins are tagged with ubiquitin, which serves to route them to the proteasome for degradation. A role for this pathway in

*To whom correspondence should be addressed. Tel: + 44 01223 762608; Fax: + 44 01223 331206; Email: dcr1000@cus.cam.ac.uk

poly(Q) diseases is supported by the observations that these proteins are ubiquitinated and that the aggregates are associated with proteasomal subunits (4,6,11). Furthermore, we and others have reported that inhibition of the proteasome with lactacystin, or its more potent derivative beta-lactone, resulted in increased inclusion formation (4,12,13). However, subsequent to many of these studies, recent reports have shown that lactacystin also inhibits cathepsin A activity of the lysosome and thus may not be proteasome-specific (14,15).

The lysosomes, often considered as non-specific systems for protein degradation, have recently also been shown to be able to selectively receive and degrade certain intracellular proteins. The process of bulk degradation of cytoplasmic proteins or organelles in the lytic compartment is termed autophagy. It involves the formation of double-membrane structures called autophagosomes or autophagic vacuoles, which fuse with the primary lysosomes, where their contents are degraded and then either disposed off or recycled back to the cell (16). There are many lines of evidence implicating autophagy in PCDs. Huntingtin-containing vacuoles display the ultrastructural features of early and late autophagosomes, and huntingtin-enriched cytoplasmic vacuoles formed in vitro contain the lysosomal enzyme cathepsin D (17). Several studies have shown that OPMD is characterized by the presence of rimmed vacuoles, and elevated levels of autophagic structures were found to be associated with Parkinson's disease (18). However, none of these studies have directly tested if autophagy is involved in degradation of any aggregate-prone protein.

In this study, we have used enhanced green fluorescent protein (EGFP) tagged to exon 1 of the HD gene with expanded repeats and EGFP tagged to 19 alanines as models for aggregate-prone proteins due to codon reiteration mutations. Inhibition of autophagy at different steps increased the levels and rate of aggregate formation of these proteins, while increased aggregate clearance was associated with rapamycin treatment, which stimulates autophagy. Thus, our data suggest that autophagy is a major route for the degradation of these aggregate-prone proteins.

RESULTS

Inhibition of the sequestration stage of autophagy increases aggregate formation and cell death in COS-7 cells expressing mutated HD exon 1 or polyalanine protein

In this study, we have used two aggregation-prone proteins associated with codon reiteration mutations. First, we analysed an exon 1 fragment of the HD gene with 74 or 55 glutamines (Q74/Q55) fused to EGFP. Second, the effects of perturbing autophagy on the HD constructs were compared with another aggregate-prone protein – EGFP fused to 19 alanine repeats (A19). Both the Q74/55 and A19 constructs form aggregates and are associated with increased cell death, compared with Q23 and A7 constructs or empty EGFP vectors, which do not form any aggregates under the same expression conditions (4,9).

We initially examined the role of autophagy in degrading these proteins using the specific inhibitor 3-methyladenine (3-MA) (19). 3-MA inhibits autophagy at the sequestration stage, where a double-membrane structure forms around a portion of the cytosol and sequesters it from the rest of cytoplasm to form the autophagosome (16). 3-MA treatment resulted in an obvious change in the appearance of the aggregates formed by Q74 or A19 constructs transiently transfected into COS-7 cells (Fig. 1A). In cells with aggregates, 3-MA increased their apparent size and number (Fig. 1A). 3-MA treatment also increased the proportions of Q74- or A19-expressing cells with aggregates, and this was accompanied by an increase in cell death (Fig. 1B). 3-MA did not cause aggregate formation in COS-7 cells expressing EGFP-tagged wild-type HD exon 1 protein with 23 glutamine repeats or an EGFP–polyalanine protein with 7 repeats (data not shown). Identical aggregate phenotypes and similar significant increases in the proportions of Q74- and A19-transfected cells with aggregates or cell death were observed after treatment with another inhibitor of the sequestration stage of autophagy, N^6,N^6-dimethyladenosine (DMA) (19) (results not shown). Similar results were obtained with Q55 and Q74 (data not shown). The increased aggregation of Q74 and A19 caused by 3-MA was associated with an increase in the levels of the transgene protein (Fig. 1C; we obtain about 80% transfection efficiency in COS-7 cells). 3-MA treatment did not cause any change in the levels of empty EGFP transfected in the same way into COS-7 cells. However, it did cause an increase in the levels of an HA-tagged version of the same HD exon 1 fragment with Q74 (Fig. 1D, E). Thus, the 3-MA is not acting simply on the EGFP part of the fusion proteins that we have studied. These results suggest that inhibiting the sequestration stage of autophagy results in increased levels of the mutant proteins, thereby enhancing their aggregation.

Inhibition of autophagosome–lysosome fusion increases aggregation of Q74 or A19 in COS-7 cells

After the sequestration step, the autophagosome needs to fuse with the lysosome in order for its contents to be degraded. We tested this next step with the vacuolar ATPase inhibitor bafilomycin A1 (BafA1), which interferes with the autophagosome–lysosome fusion step, possibly because lysosomal acidification is required for this fusion (20). As seen in Figure 2A and B, treatment with BafA1 resulted in a change in aggregate morphology (increased size) and increased the proportions of transfected Q74 or A19 COS-7 cells with aggregates, similar to the results with 3-MA (Fig. 1A, B). This was also accompanied by an increase in cell death in these cells.

3-MA and BafA1 decrease turnover of Q74 aggregates in stable inducible PC12 cells

In order to investigate the role of autophagy in degrading misfolded proteins in another cell line, we used a stable doxycycline-inducible PC12 cell line expressing EGFP-tagged HD exon 1 with Q74, which we have previously characterized (21). This cell line also allowed us to specifically switch off transgene expression by removing doxycycline from the medium, without interfering with ongoing cellular protein synthesis. This is important because autophagy is protein-synthesis-dependent (22,23) (see below). The stable lines were
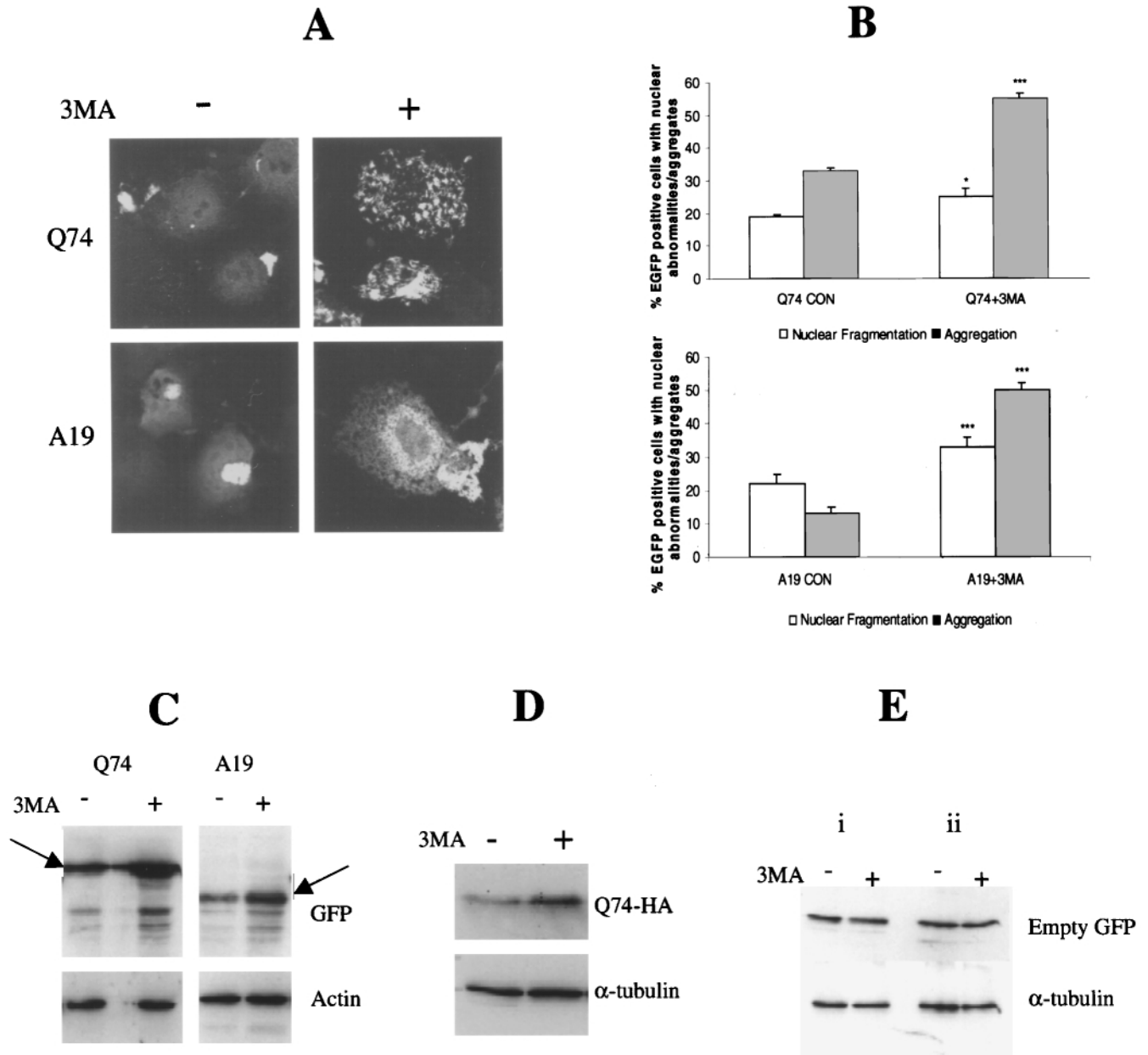
Figure 1. 3-methyladenine (3-MA) increases aggregation and cell death in transient transfection of COS-7 cells expressing Q74 or A19. (A) Immunofluorescence images of aggregate-containing COS-7 cells 48 hours after transfection with Q74 (top) or A19 (bottom), without (−) or with (+) treatment with 10 mM 3-MA for 15 hours prior to fixing. (B) Percentages of Q74- or A19-transfected COS-7 cells with aggregates and abnormal nuclei under conditions described above for (A). (C) Western blot of lysates from Q74- or A19-transfected COS-7 cells with (+) or without (−) 3-MA treatment, as in (A). The top panel shows the soluble fraction of Q74 or A19 protein marked by an arrow detected using anti-GFP antibody and the bottom panel is the actin. (D) Western blot of HA-tagged Q74 detected by an anti-HA antibody with (+) or without (−) 3-MA (top), (conditions as in A). The bottom panel shows α-tubulin as a control. (E) Western blot analysis of lysates from COS-7 cells transfected with EGFP alone with (+) or without (−) 3-MA. (i) and (ii) represent two different sets of experiments with the EGFP band seen in the top panel and α-tubulin in the bottom.

induced for 8 hours (8 h ON) and expression was then switched off by removing doxycycline from the medium for the next 24, 48 or 72 hours, which we have called 24, 48 or 72 h OFF. The proportion of cells with aggregates peaked at 24 h OFF and subsequently was reduced over time (Fig. 3A). The peak at 24 h OFF may reflect a delay in the washout of doxycycline, or may be a function of the kinetics of aggregation – aggregation occurs after a relatively long lag phase. Western blot analysis of

these cells shows aggregates as a high-molecular-weight band in the stack at 48 h OFF, but these have largely disappeared at 72 h OFF (as seen in Fig. 3B, 48 h OFF and 72 h OFF). The reduction in aggregates from 24 to 48 to 72 hours correlated with a reduction in the amounts of soluble protein (Figs 3 and 5B). In order to test the effects of 3-MA or BafA1 on the turnover of aggregates, the cells were induced for 8 hours, then switched off for 48 or 72 hours with these treatments. As clearly
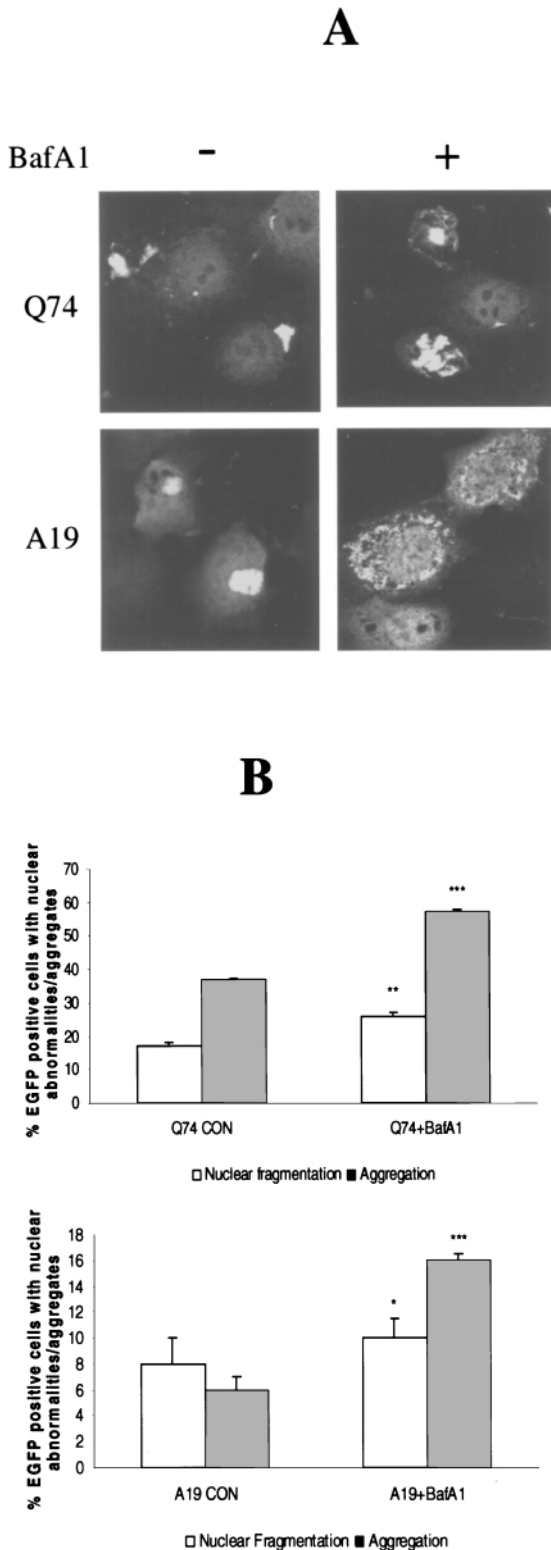
A

B

Figure 2. Bafilomycin A1 (BafA1) increases aggregation and cell death in COS-7 cells expressing Q74 or A19. (A) Immunofluorescence images of COS-7 cells, showing aggregates of Q74 (top) or A19 (bottom) after 48 hours of transfection with (+) or without (−) 200 nM BafA1. BafA1 was added 15 hours before fixing cells. (B) Percentages of EGFP-positive cells with Q74 or A19 aggregates and abnormal nuclear morphology with and without BafA1 (conditions as in A). CON, control.

seen in Figure 3A, treatment with either of the inhibitors (72 h OFF + 3-MA and 72 h OFF + BafA1) resulted in increased numbers of aggregate-containing cells when compared with time-matched control (72 h OFF). This is also supported by the results of the western blot – Figure 3B shows that 3-MA or BafA1 resulted in increased aggregate formation, as seen by the high-molecular-weight fraction on the stack, which persists even 72 hours after switching off the expression (compared with time-matched untreated controls). These results suggest that inhibiting the autophagy–lysosomal pathway might interfere with the degradation of the mutant HD exon 1 protein/aggregates, consistent with the transient transfection experiments in COS-7 cells.

Induction of autophagy decreases aggregation and cell death in mammalian cells expressing Q74 and A19

Since inhibition of autophagy resulted in increased levels as well as increased aggregation of Q74 or A19, we next wanted to examine whether induction of autophagy with an antifungal macrolide antibiotic, rapamycin, had the opposite effect. Treatment with rapamycin resulted in a decrease in the proportions of aggregate-containing cells and cell death in COS-7 cells expressing A19, but no significant change was observed in cells expressing Q74 after 48 hours of transfection (Fig. 4A). The aggregates formed by Q74 at 48 hours were much larger than at 24 hours, and the aggregation rate in the untreated Q74 cells was higher when compared with the A19 aggregates (Fig. 4A). Thus, Q74 may aggregate more rapidly than A19 and more quickly form highly stable structures that are relatively resistant to rapamycin (autophagy). We therefore repeated the experiment at 24 hours post transfection with Q74, when fewer cells have formed aggregates (Fig. 4B). Under these conditions, rapamycin reduced the proportions of cells with aggregates and cell death (Fig. 4B). We performed similar experiments in the stable inducible PC12 cell line expressing EGFP-Q74. The cells were induced for 8 hours and then expression was switched off for 24, 48 or 72 hours, with or without rapamycin treatment. The rate at which protein clearance occurred was measured by the loss of fluorescence and also by comparing the protein levels on a western blot. As seen in Figure 5A, treatment with rapamycin resulted in a loss of fluorescence [(i) 72 h OFF + Rap versus. 72 h OFF] and also a decrease in protein levels on a western blot when compared with the untreated control (Figs 3B and 5B). Since rapamycin is a slow inhibitor of protein synthesis, we checked whether inhibition of protein synthesis by treatment with cycloheximide had a similar effect on Q74 clearance and aggregation. In contrast to rapamycin, cycloheximide alone increased cell fluorescence and the number of visible aggregates (Fig. 5A, 72 h OFF + cycloheximide). Indeed, cycloheximide abrogated the effect of rapamycin on these cells (Fig. 5A, 72 h OFF+ Rap and Cycloheximide), consistent with the observation that cycloheximide causes a drastic reduction in autophagy-induced protein degradation (22,23).

Effect of proteasome inhibition by epoxomicin

We and others have previously reported that inhibition of the proteasome with lactacystin results in increased aggregation of
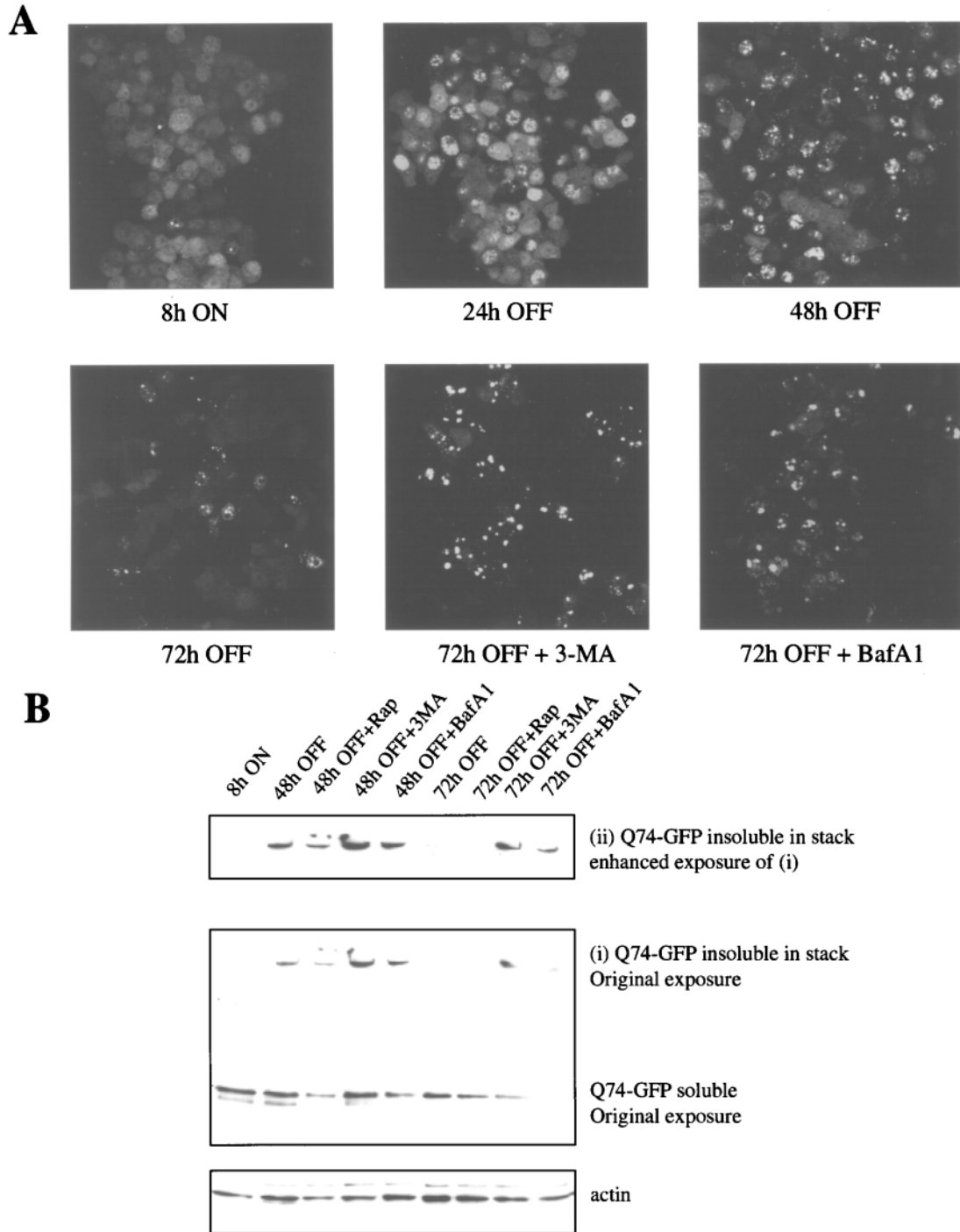
Figure 3. 3-MA and BafA1 decrease aggregate clearance in a stable inducible PC12 cell line expressing Q74. (A) Immunofluorescence images of the PC12 cells stably expressing Q74. The cells were induced with doxycycline for 8 hours (8 h ON) and the expression was then switched off by the removal of doxycycline from the medium for 24, 48 or 72 hours (24, 48 or 72 h OFF). 3-MA or BafA1 treatments (72 h OFF + 3-MA or BafA1) were done for 72 hours concurrently with turning off the expression, and the figures show the presence of many aggregate containing cells when compared with time-matched control (72 h OFF). (B) Western blot analysis of PC12 cell lysates stably expressing Q74 induced for 8 hours (8 h ON) and switched off for 48 or 72 hours, either without treatment or after treatment with 0.2 μg/ml rapamycin (Rap), 10 mM 3-MA or 200 nM BafA1. The treatments were initiated concurrently with switching off the expression. Note that the high-molecular-weight bands on the stack [marked by (i) and the same at a longer exposure marked by (ii)] corresponding to the aggregates in 48 h OFF and 48 h OFF + Rap disappear in 72 h OFF and 72 h OFF + Rap. On the other hand, those in 48 h OFF + 3-MA or BafA1 persist in 72 h OFF + 3-MA or BafA1, implying that the clearance of Q74 is affected by the treatment with 3-MA or BafA1, causing them to aggregate. Treatment with rapamycin reduces both the aggregates (48 h OFF + Rap) and the level of the soluble protein (48 or 72 h OFF + Rap). The immunofluorescent images were all taken at the same intensity.
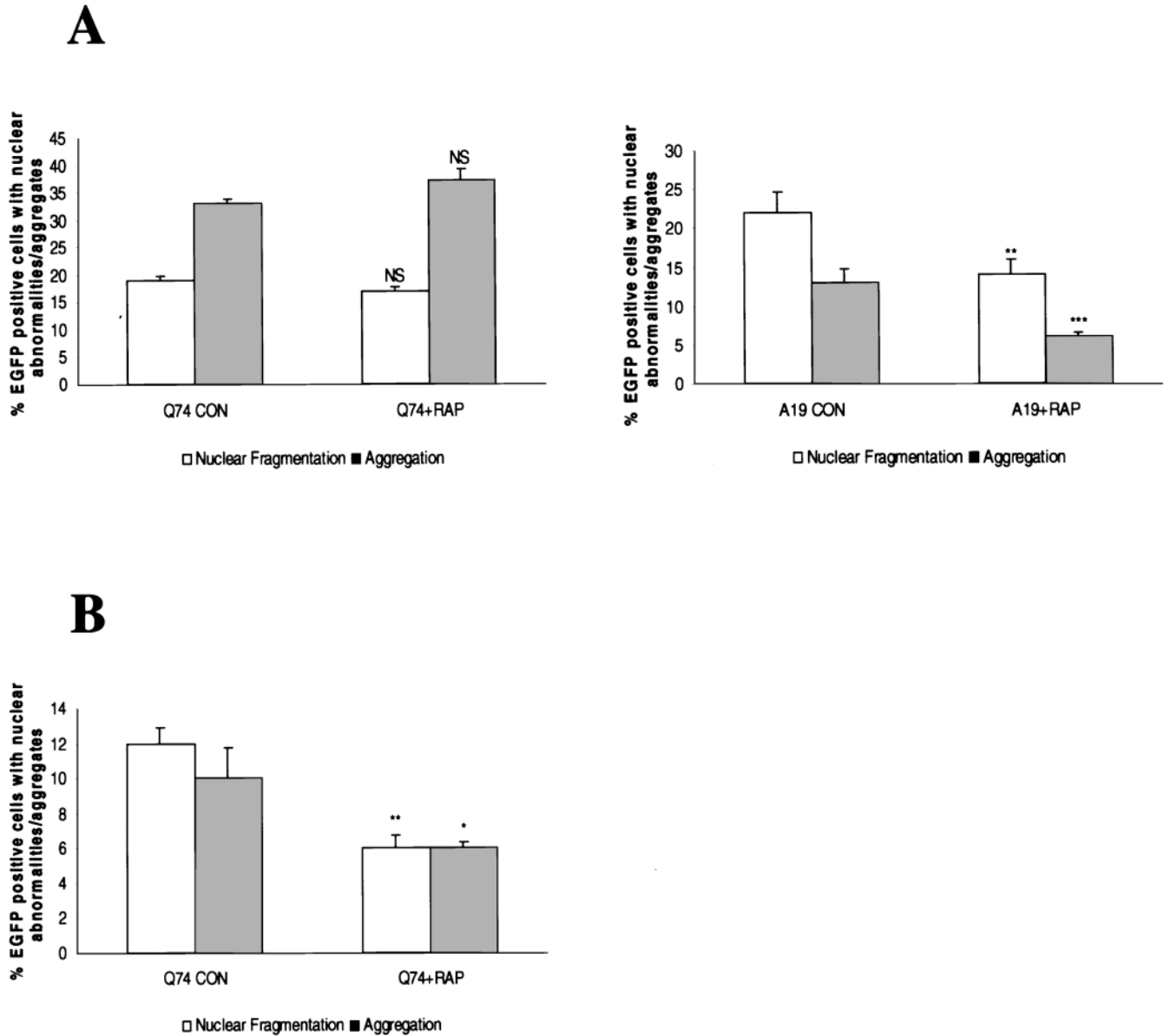
A

B

Figure 4. Rapamycin reduces aggregation and cell death in COS-7 cells expressing Q74 or A19. (A) Percentages of Q74 or A19-transfected COS-7 cells after 48 hours with aggregates and abnormal nuclear morphology. CON, control; RAP, rapamycin (at $0.2 \mu g/ml$ final concentration added 15 hours prior to fixing). (B) Percentages of EGFP-positive COS-7 cells with Q74 aggregates and cell death after 24 hours of transfection. Here rapamycin was added as in (A).

Q74 in transient transfection experiments (4,12,13). However, recent studies have reported that lactacystin can also inhibit cathepsin A activity, and thus may interfere with lysosomal function (14,15). So we tested the effect of epoxomicin, a potent and apparently selective proteasome inhibitor (24), in COS-7 cells expressing Q74 or A19 and in PC12 cells stably expressing Q74. The immunofluorescence images in Figure 6A reveal a change in morphology of Q74 or A19 aggregates in COS-7 cells treated with epoxomicin. As we described previously with Q74 and lactacystin (4), there were more aggregates in each aggregate-containing cell. However, epoxomicin treatment did not affect the proportion of Q74-

expressing cells with aggregates or apoptotic nuclear morphology. This is in contrast to lactacystin, which we previously showed increased the proportion of Q74-expressing cells with aggregates (4). However, epoxomicin did increase the proportion of COS-7 cells expressing A19 with aggregates (Fig. 6B). PC12 cells stably expressing Q74 were induced for 8 hours, and expression was switched off for 48 hours without (48 h OFF) or with treatment with epoxomicin (48 h OFF + Epox) or lactacystin (48 h OFF + Lac). Surprisingly, epoxomicin resulted in a reduction in the number of aggregate-containing cells when compared with control, even though it caused an obvious increase in cellular fluorescence. This was confirmed
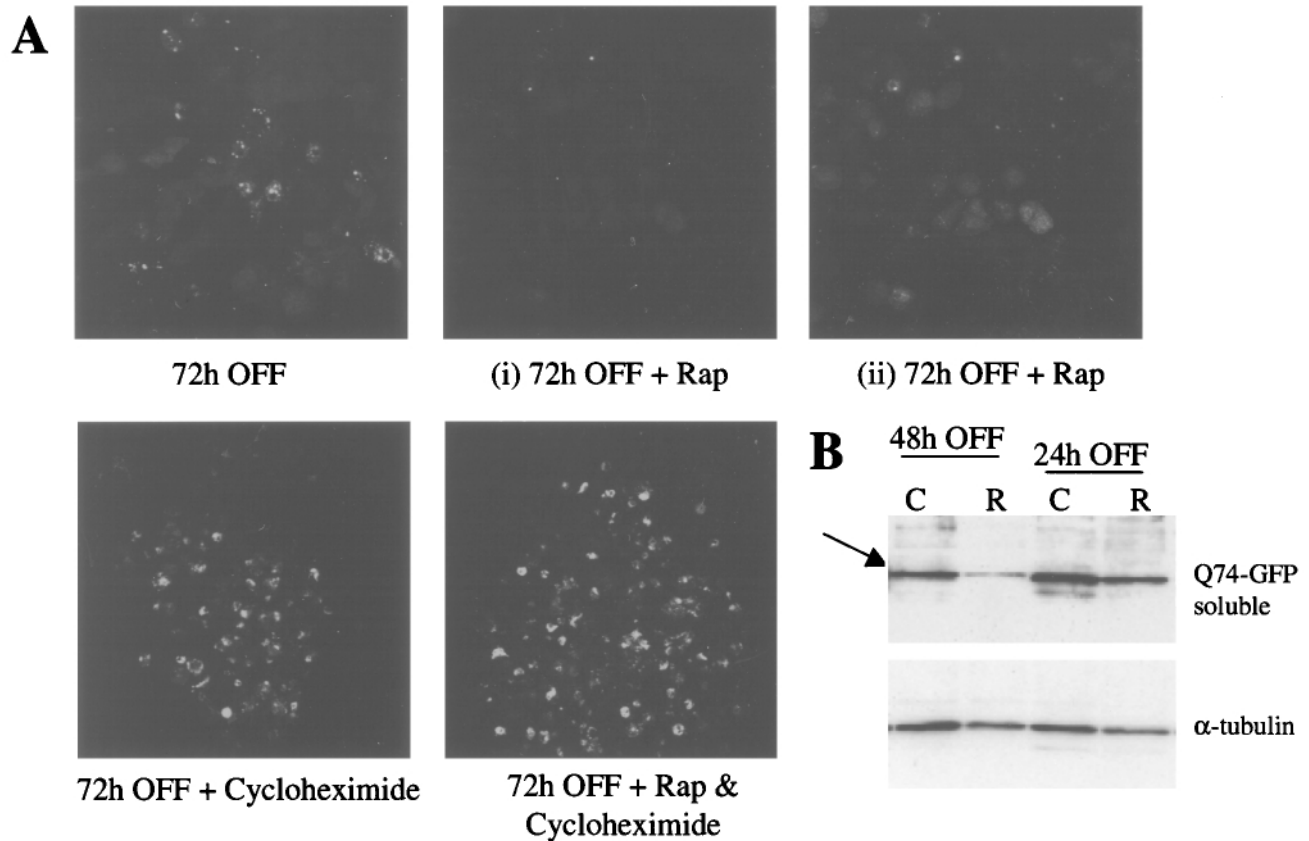
Figure 5. Rapamycin reduces aggregation of Q74 in stable PC12 cell line. PC12 cells expressing Q74 were induced for 8 hours, and the expression was turned off for 72 hours (72 h OFF) without or with rapamycin (72 h OFF + Rap). All slides were scanned at the same fluorescent intensity, except for (ii) 72 h OFF + Rap, which was scanned with enhanced fluorescence intensity. Note the decreased fluorescence on rapamycin treatment. Immunofluorescence figures on the bottom panel shows the 72 h OFF PC12 cells with cycloheximide (at 10 µg/ml concentration) or with cycloheximide and rapamycin together. Treatment with cycloheximide or cycloheximide plus rapamycin did not reduce the fluorescence. (B) Western blot analysis of PC12 24 h OFF or 48 h OFF with (R) or without (C) rapamycin using anti-GFP antibody (top panel) and anti-tubulin antibody (bottom).

by western blot analysis of epoxomicin-treated cells, which revealed a marked increase in the soluble fraction but failed to detect any high-molecular-weight bands corresponding to aggregates (Fig. 7B). This is in contrast to lactacystin, which increased Q74 aggregation immunocytochemically (Fig. 7A, 48 h OFF + Lac) and both the soluble and insoluble fractions on western blots (Fig. 7C). We also noted a marked increase in heat shock protein 70 (HSP70) on epoxomicin treatment (Fig. 7B), consistent with inhibition of the proteasome (25,26).

DISCUSSION

In this study, we have used poly(Q) and poly(A) expansions as models for aggregate-prone proteins caused by codon reiteration mutations. We tested if autophagy plays a major role in the degradation of such proteins, since previous studies had shown increased numbers of autophagic vesicles in HD cell models and in vivo. Furthermore, some huntingtin was detected within autophagosomes in an HD cell model (17). Our data suggest that autophagy is indeed involved in the degradation of our

model proteins, since these accumulated when cells were treated with different inhibitors acting at distinct stages of the autophagy–lysosome pathway, in two different cell lines. These inhibitors are frequently used to test the role of autophagy in different contexts. Furthermore, rapamycin, which stimulates autophagy (23), enhanced the clearance of our aggregate-prone proteins. Rapamycin also reduced the appearance of aggregates and the cell death associated with the poly(Q) and poly(A) expansions. Rapamycin potently inhibits downstream signalling from the target of rapamycin (TOR) proteins. Phophorylation of ribosomal protein S6 strongly correlates with the inhibition of macroautophagy, and the activity of p70S6 kinase is regulated by mTOR kinase (18). Inhibition of phosphorylation resulting from inactivation of mTOR by treatment with rapamycin induces autophagy; however, the details of the regulatory mechanism are not understood. Since rapamycin is already used clinically (e.g. for the treatment of transplant rejection) (27,28), this drug or related analogues may be suitable candidates for therapeutic investigation in HD and related diseases.

Mutant huntingtin clearance is likely to be modulated by a number of factors, since the protein is proteolytically cleaved at
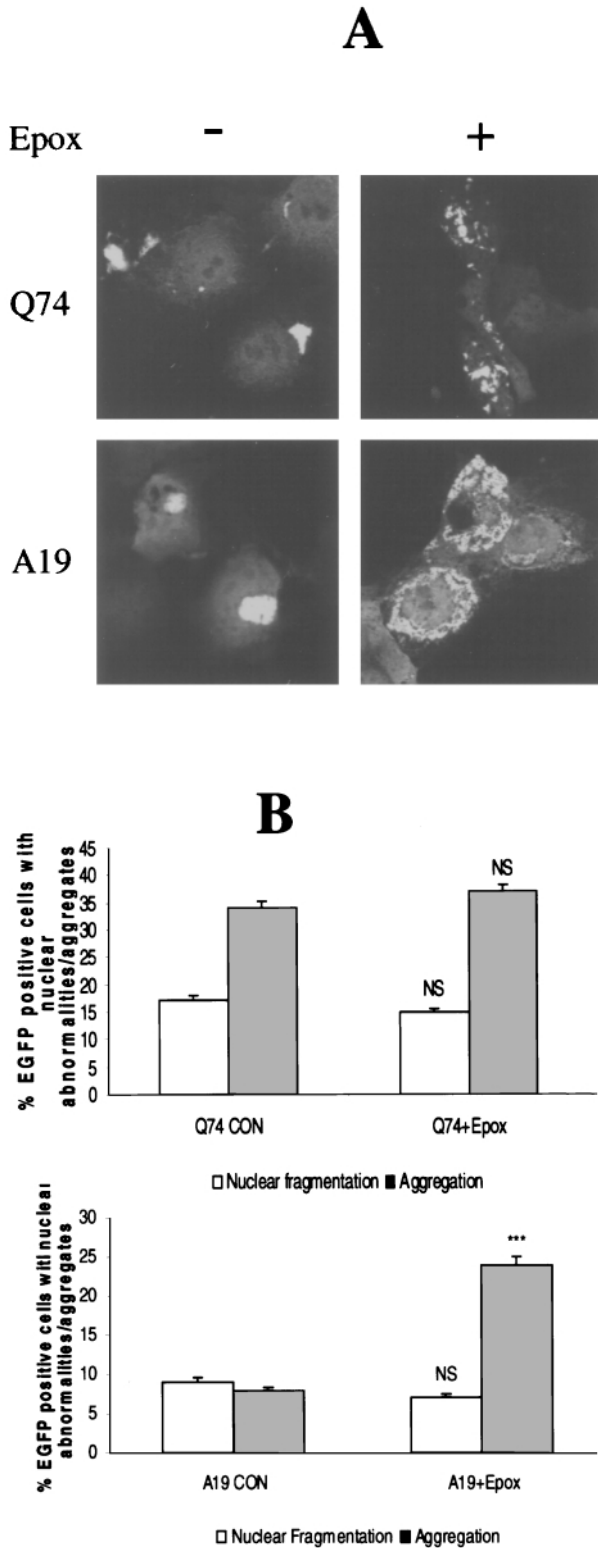
A

B

Figure 6. Effect of epoxomicin on COS-7 cells transfected with Q74 or A19. (A) Immunofluorescence images of COS-7 cells, showing aggregates transfected for 48 hours with Q74 (top) or A19 (bottom) with (+) or without (−) 10 μM epoxomicin added 15 hours before fixing the cells. (B Percentage of Q74- or A19-transfected cells with aggregates and nuclear abnormalities with and without epoxomicin (conditions as above). CON, control, Epox, epoxomicin.

a multiple sites by enzymes recognizing caspase recognition sequences and by calpains (5,29,30). The mutant protein appears to be cleaved to form an N-terminal fragment containing the glutamine repeats, which is more toxic and which is also prone to aggregation, compared with the full-length protein, which does not show obvious toxicity or aggregation. It is possible that the efficiency of the cleavage steps and the clearance rate of intermediate fragments may also influence the steady-state levels of aggregate-prone huntingtin fragments. However, this model is controversial and the exact nature of the cleavage fragments has not been elucidated (31). In the absence of clarity regarding the nature of the aggregate-prone species, we have used huntingtin exon 1 fragments and GFP–poly(A) constructs with expanded repeats as generic models for aggregate-prone proteins. When the aggregate-prone huntingtin fragments are characterized, it will be important to confirm that their clearance is mediated by autophagy and/or the ubiquitin–proteasome pathway – the multiple cleavage steps would create considerable difficulties for the approaches used in this paper, if we started with full-length huntingtin.

Our data confirm the previous data from the conditional HD mouse model (32) that the aggregates are not stable entities, since they gradually disappeared over time in the PC12 cells after tetracycline was removed. This suggests that either the aggregates are solubilized or they are directly degraded in these cells. There is probably a two-way traffic between soluble and aggregated protein, and the half-lives of the protein will probably differ in aggregates compared with the soluble state. Thus, traditional pulse-chase experiments may be very difficult to interpret because of a lack of first-order kinetics, which would arise when there are differential amounts of the soluble versus insoluble aggregates at different time points. The ability to switch expression on and off in our inducible system has been a valuable tool to allow us to analyse protein clearance. It had the additional advantage of not requiring the use of protein synthesis inhibitors such as cycloheximide, which we have confirmed interferes with autophagy-mediated protein clearance.

While our data strongly implicate autophagy in the clearance of our aggregate-prone proteins, we have also re-examined the role of the proteasome. Virtually all studies testing the role of the proteasome in poly(Q) disease protein clearance have used lactacystin or the derivative beta-lactone. However, recent studies have shown that lactacystin may also affect lysosomal function (14,15). Here we used epoxomicin to inhibit the proteasome and found that it increased aggregation of the polyA protein but did not increase aggregation or cell death in poly(Q)-expressing COS-7 cells. This contrasts with our previous experiments with lactacystin, which clearly increased the aggregate number of poly(Q) protein in transient transfection models, which we have confirmed in this study (data not shown). We examined the differences between lactacystin and epoxomicin further in PC12 stable inducible cells after removal of doxycycline from the media, i.e. under conditions where the stimulus for the synthesis of the poly(Q) transgene was removed. Both lactacystin and epoxomicin increased the levels of soluble poly(Q) protein and increased the amount of cellular fluorescence of these EGFP-tagged transgenes. Thus, these proteins are probably cleared by the proteasome, as well as by
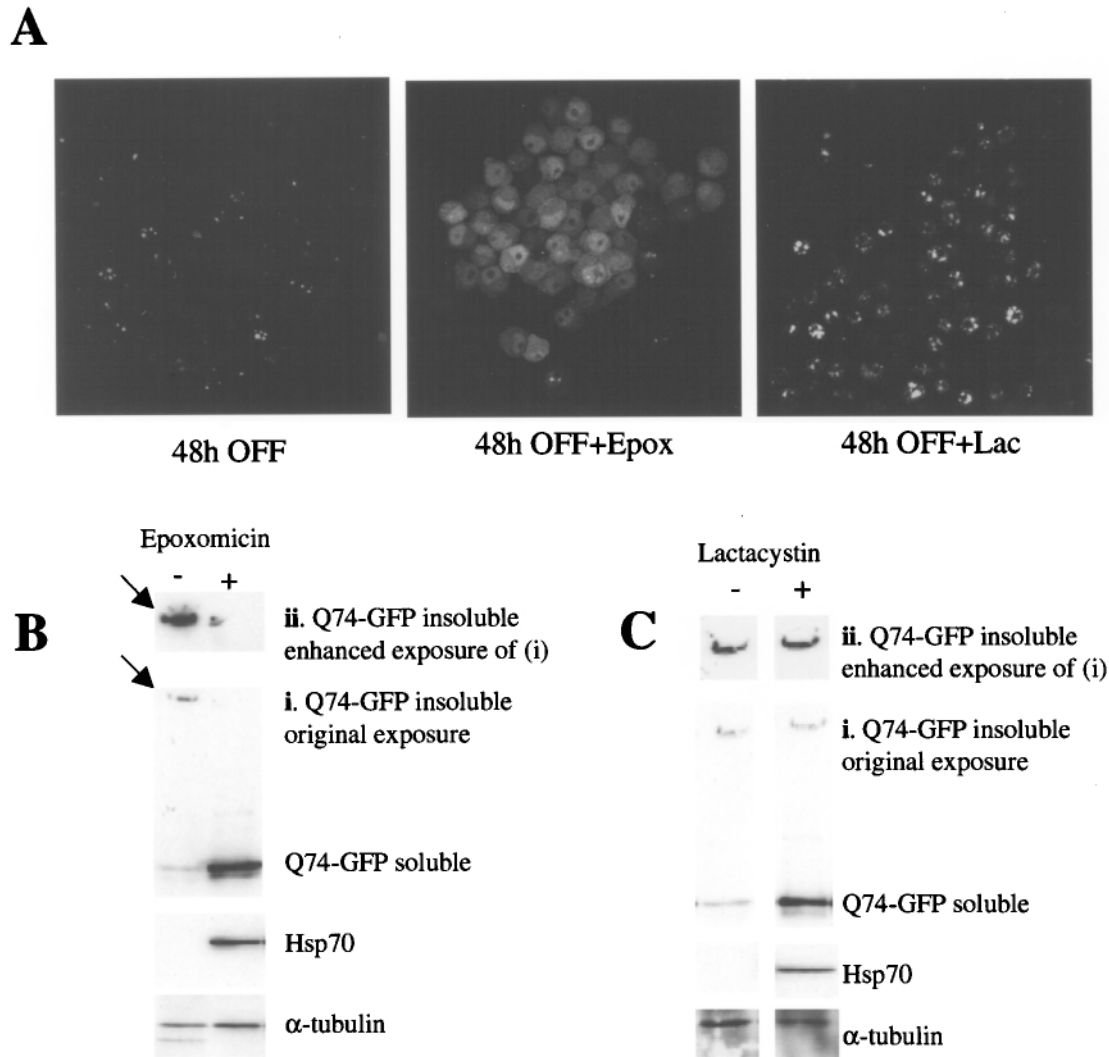
Figure 7. Epoxomicin prevents aggregation in PC12 cells stably expressing Q74. (A) Immunofluorescence images of PC12 cells expressing Q74 switched on for 8 hours. The expression was stopped for 48 hours without (48 h OFF) or with epoxomicin at 10 μM (48 h OFF + Epoxomicin) or with lactacystin at 10 μM (48 h OFF + Lactacystin) added concurrently with switching off the expression. Note the loss of aggregate-containing cells on epoxomicin treatment, but not with lactacystin. (B) Western blot analysis of epoxomicin treated (+) and untreated (−) cell lysates probed for GFP, HSP70 and α-tubulin. (i) shows the insoluble Q74 protein with anti-GFP antibody in the gel stack and (ii) is the enhanced exposure of (i). (C) Western blot analysis of lactacystin-treated (+) and untreated control (−), showing increased levels of soluble protein on lactacystin treatment; this also shows the presence of insoluble aggregates on stack in (i) using anti-GFP antibody. (ii) is the enhanced exposure of (i). The bottom panel shows the α-tubulin as a loading control. Note the reduced amount of α-tubulin (i.e. overall loading) in lactacystin-treated versus control cell lysate.

autophagy. However, in contrast to lactacystin, which dramatically increased the number of aggregate-containing PC12 cells, epoxomicin treatment reduced the appearance of aggregates in these cells, compared with controls. The reasons for this discrepancy are not clear. Epoxomicin appears to be specific for the proteasome (at least up to 50 μM) (24), while lactacystin may interfere with both proteasomal and autophagy–lysosomal pathways (14,15). Epoxomicin and lactacystin affect the various proteasomal proteases differently, and these inhibitors may also have other unknown effects on pathways relevant to aggregate formation. Thus, different spectra of unrelated cellular proteins may accumulate with these treatments, and some of these that accumulate with epoxomicin may directly prevent aggregate formation. Such proteins may include chaperones and heat-shock proteins. Both epoxomicin and lactacystin cause a dramatic heat shock response.

HSP70 may be directly anti-apoptotic in poly(Q) diseases, as distinct from simply working as a chaperone to reduce aggregate formation (33,34). Thus, it is not possible to simply extrapolate a relationship between cell death and aggregate formation, or lack thereof, under conditions of proteasomal inhibition, where this protein is induced.

In conclusion, our data reveal an alternative route for the degradation of our model aggregate-prone proteins. While the traditional view was that the ubiquitin–proteasome pathway largely clears such proteins, it is possible that the autophagy–lysosomal route may be of similar importance, at least for certain proteins.

MATERIALS AND METHODS

Plasmids used

Mammalian expression vectors comprising EGFP (pEGFP-C1, Clontech) fused at its C terminus with an HD gene exon 1 fragment with 74 polyQ repeats (Q74) or a poly(A) stretch of 19 repeats (A19) were described and characterized previously (9,10). A Haemagglutinin-tagged HD gene exon 1 fragment with 74 poly(Q) repeats in pHM6 vector (Q74-HA) was also used (10). The PC12 stable lines expressing exon 1 of the huntingtin gene were made by Dr Andreas Wyttenbach in our laboratory and were described in (21).

Mammalian cell culture and transfection

African green monkey kidney cells (COS-7) were grown in Dulbecco's modified eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate at 37°C, 5% CO_2. The cells were grown on coverslips in six-well plates for immunofluorescence analysis, or were directly grown in six-well plates to 60–80% confluency for 24 hours for western blot analysis. Transfection was done using LipofectAMINE reagent (Invitrogen) using the manufacturer's protocol. The transfection mixture was replaced by normal culture medium after 5 hours' incubation at 37°C and the transfected cells were analysed by immunofluorescence or immunoblot 48 hours after transfection. The cells were left untreated or treated with 10 mM 3-methyladenine (3-MA, Sigma), 0.5 mM N^6,N^6,dimethyladenosine (DMA, Sigma), 0.2 μg/ml rapamycin (Sigma), 200 nM bafilomycin A1 (BafA1, Sigma), 10 μM lactacystin (Sigma) or 10 μM epoxomicin (Affinity research products Ltd) for 15 hours before fixation for immunofluorescence or processing for western blots. DMA, BafA1, epoxomicin and rapamycin were dissolved in DMSO, while 3-MA and lactacystin were in water. Equal amounts of water or DMSO were added to the untreated controls, where relevant. The cells on coverslips were rinsed with $1 \times$ PBS, fixed with 4% paraformaldehyde in $1 \times$ PBS for 20 minutes and mounted in antifadent supplemented with 4′,6-diamidino-2-phenylindole (DAPI, 3 μg/ml, Sigma) to allow visualization of nuclear morphology. The PC12 stable cells were maintained at 75 μg/ml hygromycin in standard medium consisting of high-glucose DMEM (Sigma) with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine (Invitrogen), 10% heat-inactivated horse serum (Invitrogen), 5% Tet-approved fetal bovine serum (FBS) (Clontech) and 100 μg/ml G418 (Invitrogen) at 37°C, 10% CO_2. The cells were seeded at $1–2 \times 10^5$ per well in 6-well plates and were induced with 1 μg/ml doxycycline (Sigma) for 8 hours. The expression of transgenes was switched off by removing doxycycline from the medium. Cells were either left untreated or treated with 3-MA, BafA1, epoxomicin, lactacystin, rapamycin, 10 μg/ml cycloheximide (Sigma) or cycloheximide plus rapamycin at the concentrations specified above for 24, 48 or 72 hours, and the medium with the inhibitors/activator was changed every 24 hours. The cells were then scraped off from the wells into 1.5 ml Eppendorf tubes, pelleted at 8000 r.p.m. in an Eppendorf 5415D benchtop microcentrifuge and washed twice with $1 \times$ PBS. They were either fixed with 4% paraformaldehyde for 20 minutes, mounted in DAPI over coverslips on glass slides or processed for western blot analysis.

Western blot analysis

The pellets for westerns from COS-7 or PC12 cells were lysed on ice in Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) for 30 minutes in the presence of protease inhibitors (Roche Diagnostics). The lysates were subjected to SDS–PAGE (10%) electrophoresis and proteins transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). The primary antibodies used include mouse monoclonal anti-GFP antibody (8362-1, Clontech) at 1 : 2000, rabbit monoclonal anti-actin antibody (A2066, Sigma) at 1 : 3000, mouse monoclonal anti-tubulin antibody (Clone DM 1A, Sigma) at 1 : 1000 and mouse monoclonal anti-HA antibody (Covance) at 1 : 1000. Blots were probed with horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Bio Rad) at 1 : 2000. Bands were visualized using the ECL detection reagent (Amersham).

Quantification of aggregate formation and abnormal cell nuclei

Aggregate formation and nuclear morphology were assessed using a fluorescence microscope. 200 EGFP-positive COS-7 cells were selected and the proportion of cells with aggregates was counted. Aggregates are described in (9,10). Cells were considered dead if the DAPI-stained nuclei showed apoptotic morphology (fragmentation or pyknosis). Pyknotic nuclei are typically less than 50% of the diameter of normal nuclei and show obvious increased DAPI intensity. We have demonstrated that these criteria are specific for cell death, since they show a very high correlation with propidium iodide staining in live cells (A. Wyttenbach and D.C. Rubinsztein, unpublished data). Furthermore, these nuclear abnormalities are reversed with caspase inhibitors (4). Analysis was performed with the observer blinded to the identity of the slides, and all experiments reported in the figures were done in triplicate at least twice.

Statistical analysis

The P-values were determined by unconditional logistical regression analysis using the general loglinear analysis option of SPSS Ver. 6.1. software (SPSS, Chicago).

ACKNOWLEDGEMENTS

REFERENCES

1. Paulson, H.L. (1999) Protein fates in neurodegenerative proteinopathies: polyglutamine diseases join the (mis)fold. Am. J. Hum. Genet., 64, 339–345.
2. Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A., et al. (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum. Mol. Genet., 8, 397–407.
3. Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L. and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell, 90, 537–548.
4. Wyttenbach, A., Carmichael, J., Swartz, J., Furlong, R.A., Narain, Y., Rankin, J. and Rubinsztein, D.C. (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. Proc. Natl Acad. Sci. USA, 97, 2898–2903.
5. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S.U., et al. (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. Nature Genet., 18, 150–154.
6. Jana, N.R., Zemskov, E.A., Wang, G. and Nukina, N. (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum. Mol. Genet., 10, 1049–1059.
7. Brais, B., Bouchard, J.P., Xie, Y.G., Rochefort, D.L., Chretien, N., Tome, F.M., Lafreniere, R.G., Rommens, J.M., Uyama, E., Nohira, O. et al. (1998) Short GCG expansions in the PABP2 gene cause oculopharyngeal muscular dystrophy. Nature Genet., 18, 164–167.
8. Fan, X., Dion, P., Laganiere, J., Brais, B. and Rouleau, G.A. (2001) Oligomerization of polyalanine expanded PABPN1 facilitates nuclear protein aggregation that is associated with cell death. Hum. Mol. Genet., 10, 2341–2351.
9. Rankin, J., Wyttenbach, A. and Rubinsztein, D.C. (2000) Intracellular green fluorescent protein-polyalanine aggregates are associated with cell death. Biochem. J., 348, 15–19.
10. Narain, Y., Wyttenbach, A., Rankin, J., Furlong, R.A. and Rubinsztein, D.C. (1999) A molecular investigation of true dominance in Huntington's disease. J. Med. Genet., 36, 739–746.
11. Suhr, S.T., Senut, M.C., Whitelegge, J.P., Faull, K.F., Cuizon, D.B. and Gage, F.H. (2001) Identities of sequestered proteins in aggregates from cells with induced polyglutamine expression. J. Cell Biol., 153, 283–294.
12. Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H. and Wanker, E.E. (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. Mol. Biol. Cell, 12, 1393–1407.
13. Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science, 292, 1552–1555.
14. Ostrowska, H., Wojcik, C., Wilk, S., Omura, S., Kozlowski, L., Stoklosa, T., Worowski, K and Radziwon, P. (2000) Separation of cathepsin A-like enzyme and the proteasome: evidence that lactacystin/beta-lactone is not a specific inhibitor of the proteasome. Int. J. Biochem. Cell Biol., 32, 747–757.
15. Kozlowski, L., Stoklosa, T., Omura, S., Wojcik, C., Wojtukiewicz, M.Z., Worowski, K. and Ostrowska, H. (2001) Lactacystin inhibits cathepsin A activity in melanoma cell lines. Tumour Biol., 22, 211–215.
16. Klionsky, D.J. and Oshumi, Y. (1999) Vacuolar import of proteins and organelles from the cytoplasm. Annu. Rev. Cell Dev. Biol., 15, 1–32.
17. Kegel, K.B., Kim, M., Sapp, E., McIntyre, C., Castano, J.G., Aronin, N. and DiFiglia, M. (2000) Huntingtin expression stimulates endosomal-lysosomal activity, endosomal tubulation and autophagy. J. Neurosci., 20, 7268–7278.
18. Klionsky, D.J. and Emr, S.D. (2000) Autophagy as a regulated pathway of cellular degradation. Science, 290, 1717–1721.
19. Kovács, A.L., Gordon, P.B., Grotterød, E.M. and Seglen, P.O. (1998) Inhibition of hepatocytic autophagy by adenosine, adenosine analogs and AMP. Biol. Chem. 379, 1341–1347.
20. Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R. and Tashiro, Y. (1998) Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct. Funct., 23, 33–42.
21. Wyttenbach, A., Swartz, J., Kita, H., Thykjaer, T., Carmichael, J., Bradley, J., Brown, R., Maxwell, M., Schapira, A., Orntoft, T.F. et al. (2001) Polyglutamine expansions cause decreased CRE-mediated transcription and early gene expression changes prior to cell death in an inducible cell model of Huntington's disease. Hum. Mol. Genet., 10, 1829–1845.
22. Lawrence, B.P. and Brown, W.J. (1993) Inhibition of protein synthesis separates autophagic sequestration from the delivery of lysosomal enzymes. J. Cell Sci., 105, 473–480.
23. Abeliovich, H., Dunn, W.A. Jr., Kim, J. and Klionsky, D.J. (2000) Dissection of autophagosome biogenesis into distinct nucleation and expansion steps. J. Cell Biol. 151, 1025–1034.
24. Meng, L., Mohan, R., Kwok, B.H., Elofsson, M., Sin, N. and Crews, C.M. (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. Proc. Natl Acad. Sci. USA, 96, 10403–10408.
25. Kawazoe, Y., Nakai, A., Tanabe, M. and Nagata, K. (1998) Proteasome inhibition leads to the activation of all members of the heat-shock-factor family. Eur. J. Biochem., 255, 356–362.
26. Kim, D., Kim, S.H. and Li, G.C. (1999) Proteasome inhibitors MG132 and lactacystin hyperphosphorylate HSF1 and induce hsp70 and hsp27 expression. Biochem. Biophys. Res. Commun., 254, 264–268.
27. Benito, A.I., Furlong, T., Martin, P.J., Anasetti, C., Appelbaum, F.R., Doney, K., Nash, R.A., Papayannopoulou, T., Storb, R., Sullivan, K.M. et al. (2001) Sirolimus (rapamycin) for the treatment of steroid-refractory acute graft-versus-host disease1. Transplantation, 72, 1924–1929.
28. Chan, L., Gaston, R. and Hariharan, S. (2001) Evolution of immunosuppression and continued importance of acute rejection in renal transplantation. Am. J. Kidney Dis., 38, S2–S9.
29. Wellington, C.L., Singaraja, R., Ellerby, L., Savill, J., Roy, S., Leavitt, B., Cattaneo, E., Hackam, A., Sharp, A., Thornberry, N. et al. (2000) Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. J. Biol. Chem. 275, 19831–9838.
30. Kim, Y.J., Yi, Y., Sapp, E., Wang, Y., Cuiffo, B., Kegel, K.B., Qin, Z.H., Aronin, N. and DiFiglia, M. (2001) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc. Natl Acad. Sci. USA, 98, 12784–12789
31. Dyer, R.B. and McMurray, C.T. (2001) Mutant protein in Huntington disease is resistant to proteolysis in affected brain. Nat. Genet. 29, 270–278.
32. Yamamoto, A., Lucas, J.J. and Hen, R. (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell, 101, 57–66.
33. Zhou, H., Li, S.H. and Li, X.J. (2001) Chaperone suppression of cellular toxicity of huntingtin is independent of polyglutamine aggregation. J. Biol. Chem., 276, 48417–48424.
34. Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H.T., Dillmann, W.H. and Zoghbi, H.Y. (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. Hum. Mol. Genet., 10, 1511–1518.