Autosomal dominant oculopharyngeal muscular dystrophy (OPMD) is characterized pathologically by intranuclear inclusions in skeletal muscles and is caused by the expansion of a 10-alanine stretch to 12–17 alanines in the intranuclear poly(A)-binding protein 2 (PABP2). Whereas PABP2 is a major component of the inclusions in OPMD, the pathogenic mechanisms causing disease are unknown. Here we show that polyalanine expansions in PABP2 cause increased numbers of inclusions and enhance death in COS-7 cells. We observed similar increases of protein aggregation and cell death with nuclear-targeted green fluorescent protein linked to longer versus shorter polyalanine stretches. Intranuclear aggregates in our OPMD cell model were associated with heat shock protein (HSP) 40 (HDJ-1) and HSP70. Human HDJ-1, yeast hsp104, a bacterially derived GroEL minichaperone, and the chemical chaperone Me2SO reduced both aggregation and cell death in COS-7 cells. In this study, we have tested four related hypotheses. First, polyalanine expansion mutations in different protein contexts cause proteins to misfold/aggregate and kill cells. The situation in OPMD appears to have many parallels with polyglutamine diseases, raising the possibility that misfolded, aggregate-prone proteins may perturb similar pathways, irrespective of the nature of the mutation or protein context.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Expression vectors encoding fusion proteins of EGFP with wild-type bovine PABP2 (with 10 alanines; PABP2-A10) and mutated bovine PABP2 (with 17 alanines; PABP2-A17) were made in-frame with the C-terminus of EGFP. Constructs were generated with a pCAGGS expression vector. PABP2 was amplified from human- or bovine-expressed sequence, and EGFP was amplified from pEGFP-C1 (Clontech). The amplified sequences were digested with BamHI and XhoI, and the C-terminal end was ligated into the expression vector to give pCAGGS-PABP2-A10-EGFP and pCAGGS-PABP2-A17-EGFP. The vector pCAGGS-HA-1 was used to produce constructs expressing HA-1 as a fusion protein with the N-terminus of PABP2. All constructs were sequenced to ensure accuracy.

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by subcloning the respective PABP2 cDNAs from the pGM10-His/bovPABP2 wt and pGM10-His/bovPABP2 7ala prokaryotic expression vectors (kindly provided by Prof. Elmar Wahle; Halle, Germany) into the pEGFP-C1 vector (CLONTECH). pGM10-PABP2 was cut with NdeI, and the resulted 3' ends were filled. The linear plasmid was then cut with SmaI to release the respective PABP2 cDNA fragments. The PABP2 wt and PABP2 7ala fragments were purified and ligated to the Smal-BamHI sites of pEGFP-C1. All constructs were validated by DNA sequencing. Bovine PABP2 differs from its human orthologue by 2 of 306 amino acids (15).

Oligonucleotides encoding an SV40 T-antigen nuclear localization signal (NLS) were cloned into pEGFP-C1, 5'-CCGGACCCCTAGAGAACCTGATTTGATA-3' (16), were inserted into the BspEI/IgI-II sites of plasmids EGFP-A7, EGFP-A19, and EGFP-A37, expression constructs encoding EGFP with 7, 19, and 37 C-terminal polyalanine tracts (8). These constructs were named GFP-NLS-A7, GFP-NLS-A19, and GFP-NLS-A37, respectively.

**Cell Culture and Transfection**—African green monkey kidney cells (COS-7) were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate at 37 °C in 5% CO2. For transfection, cells were seeded on coverglasses at 0.6–1 × 10^5 cells/well in 6-well plates the day before transfection. A mixture of 10 μl of LipofectAMINE reagent (Invitrogen) and 2 μg of endotoxin-free plasmid DNA was added to the cells at 50–90% confluency in non-decalcified Dulbecco's modified Eagle's medium. The transfection mixture was replaced by full-serum culture medium after 5 h of incubation at 37 °C.

In co-transfection experiments, pFlag-CMV-2 (Sigma) or pFlag-CMV-2 constructs expressing full-length human HDJ-1 (12), pCDNA3.1/MCT (10), and pCDNA3.1/HSP104 (10) were co-transfected with either PABP2-A10 or PABP2-A17 constructs. Cells were transfected with 0.4 μg of GFP construct and 1.6 μg of the appropriate chaperone or empty vector DNA per well to ensure that all cells expressing the GFP fusion proteins also expressed the appropriate chaperone or empty vector DNA per well to ensure that all cells expressing the GFP fusion proteins also expressed the appropriate chaperone proteins. At 48, 72, and 96 h after transfection, cells on coverslips were washed with 1× PBS, fixed with 4% paraformaldehyde in 1× PBS for 30 min, and mounted in antifadent supplemented with 4% dimethyl-2-phenylindole (DAP, 5 μg/ml) to allow visualization of nuclear morphology.

**Western Blotting and Immunocytochemistry**—For Western blotting, cell pellets were lysed in Laemmli buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue). Lysates were subjected to SDS-polyacrylamide gel (15%) electrophoresis and transferred onto nitrocellulose membrane (Amersham Biosciences, Inc.). Primary antibodies were used at 1:1000 (rabbit polyclonal anti-PABP2 antibody; kindly provided by Dr. Elmar Wahle) or 1:2000 (mouse monoclonal anti-GFP antibody; CLONTECH). Blots were probed with peroxidase-labeled anti-rabbit or anti-mouse antibodies at 1:2000 (Amersham Biosciences, Inc.). For detection, enhanced chemiluminescence reagent (ECL, Amersham Biosciences, Inc.) was used, and the signal was detected by exposing the membrane to x-ray film.

For immunocytochemistry, COS-7 cells were grown on coverslips for 16–24 h and transfected with PABP2-A17 construct. 48 h after transfection, cells were washed and fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. After three washes with PBS, cells were incubated in blocking buffer (5% fetal bovine serum) for 30 min at room temperature. Primary antibody incubation was carried out for 90 min at room temperature. Antibodies were rabbit polyclonal anti-HDJ-1 antibody (SPA-400; StressGene Biotechnologies, Victoria, British Columbia, Canada), mouse monoclonal anti-HSP70 antibody (SPA-810; StressGene Biotechnologies), rabbit polyclonal anti-20 S proteasome antibody (Affiniti, Exeter, United Kingdom), and rabbit monoclonal anti-ubiquitin antibody (Chemicon, Harrow, United Kingdom). Antibodies were used at 1:500 for 1 h at room temperature and washed in 1× PBS three times. Then cells were incubated with either goat anti-mouse IgG or goat anti-rabbit IgG conjugated to Texas Red or Alexa 488 (Molecular Probes, Eugene, OR) for 1 h in the dark at room temperature (1:2000), washed three times in PBS, dried, and mounted in antifadent containing DAPI (see above). Samples were observed using a confocal microscope.

**Quantitation of Aggregate Formation and Abnormal Cell Nuclei**—Aggregate formation and nuclear morphology were assessed with a fluorescence microscope. 200 EGFP-expressing cells across the center region of the slides were counted (with the observer blinded to slide identity). We assessed the proportions of PABP2- or GFP-NLS-A-expressing cells that contained one or more inclusions. We considered cells to have inclusions if the GFP was abnormally concentrated and differed from the diffuse nuclear pattern seen with GFP-NLS-A7 or the nuclear speckled appearance typically seen with PABP2-A7. The nuclear morphology of COS-7 cells was analyzed using DAPI staining and was considered abnormal if the nuclei were fragmented or condensed to a small size with a much stronger fluorescence signal when compared with normal nuclei (apoptotic nuclei).

**Statistical Analysis**—Pooled estimates for the changes in inclusion formation resulting from perturbations assessed in multiple experiments were calculated as odds ratios (ORs) with 95% confidence intervals (percentage of cells expressing construct with inclusions under perturbation conditions/percentage of cells expressing construct without inclusions under perturbation conditions/percentage of cells expressing construct with inclusions under control conditions/percentage of cells expressing construct without inclusions under control conditions)). ORs were considered to be the most appropriate summary statistics because the percentage of cells with inclusions or nuclear abnormalities under the specified conditions varied between experiments on different days, whereas the relative change in the proportion of cells with inclusions or nuclear abnormalities induced by overexpression of an HSP is expected to be more constant. OR and p values were determined by unconditional logistical regression analysis using the general log linear analysis option of SPSS Version 6.1 software (SPSS, Chicago, IL).

**RESULTS**

**Polyalanine Expansions in PABP2 and Enhanced Green Fluorescent Protein Increase Protein Aggregation and Cell Death**—We have modeled OPMD in COS-7 cells by transiently overexpressing PABP2 with 10 (wild-type; PABP2-A10) or 17 alanines (mutant; PABP2-A17) fused to the C terminus of EGFP. These fusion proteins migrated on SDS-PAGE gels at the expected molecular masses, and cells transfected with the mutant and wild-type constructs expressed comparable levels of PABP2 after 48 h (Fig. 1A). Such EGFP-PABP2 (wild-type) fusions have been previously shown to localize appropriately to nuclear speckles and allow us to discriminate between wild-type transgene products and endogenous PABP2 (3). Also, Shanmugam et al. (7) studied PABP2-GFP fusions previously in COS-7 cells.

In our initial experiments, we transfected cells with 2 μg DNA/well in a 6-well plate. Whereas cells expressing PABP2-A17 formed more inclusions compared with cells expressing PABP2-A10, this was not significant (Fig. 2). Fig. 3 shows normal localization of PABP2-A10 to structures that look like the nuclear
specules described previously (3) and a typical aggregate formed by PABP2-A17. As reported previously, PABP2-containing speckles are excluded from the nucleoli (3). The aggregates formed by PABP2 exclude DAPI and thus are probably largely devoid of DNA, as is the case in vivo (6). As expected, both PABP2 constructs were localized to the nucleus (Fig. 3).

We hypothesized that wild-type PABP2 may make aggregates if it is expressed at a high concentration. Thus, we transfected cells with 0.6 or 0.2 μg of DNA to reduce the expression levels of exogenous PABP2. At these lower expression levels, PABP2-A17 made significantly more inclusions compared with PABP2-A10 (Fig. 2A). At all concentrations, PABP2-A17 caused more cell death (as judged by nuclear fragmentation and condensation, which are generally used to ascertain and quantify apoptotic nuclei) than PABP2-A10 (Fig. 2B). The lack of a neat dose response in the experiments in Fig. 2A and B may be because the experiments at different doses were not all performed at the same time.

To test whether the effects of the PABP2 polyalanine expansion were largely dependent on the protein context surrounding the mutation, we transfected cells with constructs expressing 7, 19, or 37 alanines fused to the C terminus of EGFP linked to an SV40 nuclear localization signal (GFP-NLS-A7, GFP-NLS-A19, and GFP-NLS-A37). SDS-PAGE revealed proteins of the expected molecular masses (Fig. 1B).

GFP-NLS-A7 typically produced diffuse intranuclear fluorescence, with only rare intranuclear aggregates (Figs. 2 and 4). GFP-NLS-A19 formed significantly more aggregates than GFP-NLS-A7 and localized to the nucleus in cells with and without aggregates. GFP-NLS-A37 was localized to the nucleus in cells without visible aggregates. However, in virtually all cells with GFP-NLS-A37 aggregates, these formed as a halo in the cytoplasm around the nucleus (Fig. 4). About 5–10% of GFP-NLS-A37 aggregate-containing cells had aggregates concentrated in a juxtanuclear location reminiscent of the aggregates (17). Cytoplasmic aggregates may have formed with GFP-NLS-A37 because the 37 alanines can cause rapid aggregation of the newly translated protein into polymeric forms, which are too large to pass through nuclear pores, even though these constructs have an NLS that functions effectively for soluble forms of the protein.

PABP2-A17 Aggregates Sequester Ubiquitin, Proteasome, and Heat Shock Proteins—The aggregates formed by PABP2-A17 were decorated with antibodies to ubiquitin and the 20 S proteasome component, as described in vivo for OPMD aggregates (6), suggesting that the cells recognize these proteins as being abnormally folded (Fig. 5). One of the ways that cells deal with abnormally folded and aggregate-prone proteins is to try to refold them into appropriate conformations with molecular chaperones, such as HSPs (18). The aggregates formed in our cell model were decorated by antibodies to HSP70 and HDJ-1, an HSP40 family member (Fig. 5).

Human, Yeast, Bacteria-derived, and Chemical Chaperones Reduce Aggregation and Cell Death Caused by Proteins with Expanded Polyalanine Stretches—To test whether heat shock proteins can regulate the formation of aggregates caused by polyalanine mutations, we used the human HSP40 member HDJ-1. We used this HSP because its overexpression consistently reduces the aggregation of different poly(Q) proteins (11–14). Pooled estimates for the changes in inclusion formation (or cell death) resulting from perturbations assessed in multiple experiments were calculated as ORs with 95% confidence intervals (percentage of cells expressing construct with inclusions under perturbation conditions/percentage of cells expressing construct without inclusions under control conditions)/(percentage of cells expressing construct with inclusions under control conditions/percentage of cells expressing construct without inclusions under control conditions). ORs were considered to be the most appropriate summary statistics because the percentage of cells with inclusions or nuclear abnormalities under specified conditions varied between experiments on different days, whereas the relative change in the proportion of cells with inclusions or nuclear abnormalities induced by overexpression of an HSP is expected to be more constant (10, 19). Overexpression of HDJ-1 reduced both aggregation and cell death at 48, 72, and 96 h after transfection with PABP2-A17 (Fig. 6) and at 48 h after transfection with GFP-NLS-A19 and GFP-NLS-A37 (Fig. 7). HDJ-1 is a co-chaperone for HSP70, which can directly modulate cell death pathways (20). Indeed, Zhou et al. (21) have reported that HSP70 can directly inhibit cell toxicity caused by expanded polyglutamines independent of any effect on protein aggregation. Thus, it is possible that some of the protective effects of HDJ-1 against cell death may be independent of its effects on protein misfolding/aggregation.

To address this caveat, we co-transfected cells with mutant PABP2 (or expanded GFP-polyalanine) constructs and known chaperones that are unlikely to directly affect mammalian cell death pathways. We used yeast hsp104 (for which there is no known mammalian homologue), which has previously been
shown to reduce the formation of aggregates by various proteins in yeast and by a mutant huntingtin fragment with expanded poly(Q) repeats in mammalian cells (10). We also investigated the effect of MC7, an artificial minichaperone formed by placing seven copies of the ligand-binding domain of GroEL (residues 191–376) in a ring. This mimics the organization of one of the two heptameric rings of wild-type GroEL. MC7 was made by inserting GroEL residues 191–376 in the place of part of the highly mobile loop (residues 16–33) of the bacterial co-chaperonin GroES (10, 22). GroES forms a stable structure of seven ∼10.4-kDa subunits. When analyzed by both analytical size exclusion chromatography and analytical ultracentrifugation, recombinant MC7 formed heptamers comprising seven 30-kDa subunits, and electron microscopic studies of MC7 revealed a diameter similar to that of wild-type GroEL (10, 22). The yeast and bacterial chaperone constructs described above were expressed from the mammalian expression vector pCDNA 3.1/HisB; proteins of appropriate sizes from lysates of transfected cells were detected by Western blotting, using an anti-His6 antibody (10). hsp104 and MC7 consistently reduced both aggregation and cell death at all time points tested with PABP2-A17 (Fig. 6). MC7 reduced both aggregate formation and cell death caused by GFP-NLS-A19 and GFP-NLS-A37 (Fig. 7). Whereas hsp104 reduced cell death for both GFP-NLS-A19 and GFP-NLS-A37, this chaperone did not significantly reduce the proportions of cells with GFP-NLS-A19 and GFP-NLS-A37 inclusions (Fig. 7).

To formally test the possibility that hsp104 and MC7 could
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Chaperones improving the folding of these disease proteins. If this were correct, then the protection could be ascribed to the chaperones indirectly reducing the levels of the mutant protein rather than a change from an aggregate-prone conformation to a similar level of normally folded protein. This is unlikely to be the case because overexpression of the three chaperones we studied did not appear to reduce levels of mutant PABP2 (Fig. 1C).

DISCUSSION

Our data suggest that both wild-type and mutant PABP2 proteins can aggregate at high expression levels. However, at lower levels, the mutant protein showed a significantly greater propensity for aggregation. This scenario has a precedent in the polyglutamine expansion disease spinocerebellar ataxia type 1, where at low expression levels there is aggregation of only the mutant protein with expanded repeats, whereas aggregation of the wild-type protein also occurs at high expression levels (23).

In our OPMD cell model, the expression of the mutant PABP2 construct is associated with significantly higher levels of cell death compared with the wild-type protein. There is a correlation between cell death and protein aggregation because both phenomena are reduced by mammalian, yeast, and bacterial chaperones. The bacterial and yeast chaperones we studied did not reduce cell death caused by staurosporine or H$_2$O$_2$. Thus, the protective effects they mediate in cells expressing polyalanine expansions are likely to be due to their activities as molecular chaperones improving the folding of these disease proteins. Although Me$_2$SO may have a variety of effects on cells, it mediated a reduction of inclusion formation and cell death in our PABP2 model, suggesting that Me$_2$SO may decrease the toxicity of PABP2 with expanded alanine tracts by acting as a chemical chaperone (24). While this paper was under review, Fan et al. (25) reported that deletion of domains of PABP2 required for its oligomerization reduced aggregation and cell death caused by expanded polyalanines, providing further support for an association between protein aggregation and cell death in OPMD.

We observed increases of protein aggregation and cell death with GFP-NLS-polyalanine constructs with expanded polyalanine tracts similar to those we saw with PABP2. This suggests that the toxicity of mutant PABP2 is mediated primarily by the expanded polyalanine tract and that the precise protein context may not be critical. If this is the case, then it is likely that the OPMD mutation acts via a gain of function mechanism. The reduction in cell death caused by GFP-NLS-A19 and GFP-NLS-A37 mediated by hsp104 was not mirrored by a significant reduction in aggregation. In future work, we will consider the possibility that hsp104 may modify the solubility of the aggregates without affecting the proportion of cells with aggregates, as has been described with HSF70 and polyglutamine expansions (13). We are also currently performing a detailed study to investigate which HSPs are associated with OPMD aggregates in vivo. However, the presence or absence of various HSPs on inclusions in vivo does not directly indicate whether these can modulate inclusion formation or cell death. For instance, HSF70 is not found in SCA1 inclusions in a transgenic mouse model, but its overexpression can alleviate motor function and neuropathology (26).

A gain of function mechanism for polyalanine expansion mutations in OPMD would be consistent with experiments suggesting that the capacity for PABP2 to regulate poly(A) tail elongation function is not impaired in myoblasts from OPMD patients. Further evidence against a loss of function of PABP2 in OPMD comes from studies of the Drosophila PABP2 orthologue, which lacks the first 54 residues of the mammalian proteins (27). Despite lacking the domain that is mutated in...
OPMD (starting at residue 2 of human PABP2), the *Drosophila* protein stimulates poly(A) polymerase and is able to control poly(A) length in reconstituted mammalian polyadenylation systems (27).

Two other autosomal dominant diseases, synpolydactyly and a form of cleidocranial dysplasia, are also caused by polyalanine expansions in nuclear proteins (the transcription factors HOXD13 and CBFA1) (28, 29). These diseases may also be caused by gain of function mutations because the polyalanine expansion mutations confer different or more severe phenotypes compared with null mutations. CBFA1 null mutations cause cleidocranial dysplasia, whereas the polyalanine expansion in this gene causes a different phenotype: brachydactyly of the hands/feet with only mild features of cleidocranial dysplasia (29). In addition, mice with polyalanine expansions in HOXD13 have more severe phenotypes than HOXD13 null mice (30).

How do polyalanine expansion mutations cause OPMD? An appealing model suggested by our data is that this is the direct consequence of protein aggregation. Similar findings exist for...
polyglutamine expansion diseases, where the role for protein aggregation is a subject for vigorous debate. A hallmark of most polyglutamine diseases, including Huntington’s disease, is the development of intracellular protein aggregates (inclusions) with amyloid-like β-sheet structures in the vulnerable neurons, and polyalanine tracts also form β-sheets in vitro (31, 32). Although the effects of chaperones on aggregation and cell death strongly support a correlation between the appearance of aggregates and cell death/dysfunction, they may be compatible with another theoretical model where aggregation would not necessarily be causally related to cell death. The mutant monomeric proteins may exist in two sets of conformations: a properly-folded nontoxic species and an aggregate-prone toxic form(s). If the chaperones promote the conversion of the toxic aggregate-prone monomers to the nontoxic form, then one would see a reduction of both aggregation and cell death in the presence of chaperones. This scenario may be difficult to distinguish from the model where aggregation is toxic, unless one can identify a means of distinguishing and quantifying different monomeric forms of these aggregation-prone proteins from cell models or in vivo experiments.

Irrespective of which model is correct, it is intriguing that the polyglutamine expansions we have studied show features similar to polyglutamine models. Both are associated with protein misfolding/aggregation, and it is possible that the mutant proteins may stimulate overlapping deleterious pathways in vivo, irrespective of the nature of the mutation or the protein context.

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