Genome-wide RNAi screen and in vivo protein aggregation reporters identify degradation of damaged proteins as an essential hypertonic stress response

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Choe KP, Strange K. Genome-wide RNAi screen and in vivo protein aggregation reporters identify degradation of damaged proteins as an essential hypertonic stress response. Am J Physiol Cell Physiol 295: C1488–C1498, 2008. First published October 1, 2008; doi:10.1152/ajpcell.00450.2008.—The damaging effects of hypertonic stress on cellular proteins are poorly defined, and almost nothing is known about the pathways that detect and repair hypertonicity-induced protein damage. To begin addressing these problems, we screened ~19,000 Caenorhabditis elegans genes by RNA interference (RNAi) feeding and identified 40 that are essential for survival during acute hypertonic stress. Half (20 of 40) of these genes encode proteins that function to detect, transport, and degrade damaged proteins, including components of the ubiquitin-proteasome system, endosomal sorting complexes, and lysosomes. High-molecular-weight ubiquitin conjugates increase during hypertonic stress, suggesting a global change in the ubiquitinylnation state of endogenous proteins. Using a polyglutamine-containing fluorescent reporter, we demonstrate that cell shrinkage induces rapid protein aggregation in vivo and that many of the genes that are essential for survival during hypertonic stress function to prevent accumulation of aggregated proteins. High levels of urea, a strong protein denaturant, do not cause aggregation, suggesting that factors such as macromolecular crowding also contribute to protein aggregate formation during cell shrinkage. Acclimation of C. elegans to mild hypertonicity dramatically increases the osmotic threshold for protein aggregation, demonstrating that protein aggregation-inhibiting pathways are activated by osmotic stress. Our studies demonstrate that hypertonic stress induces protein damage in vivo and that detection and degradation of damaged proteins are essential mechanisms for survival under hypertonic conditions.

Caenorhabditis elegans; kidney; polyglutamine; macromolecular crowding; cell volume

HYPERTONIC STRESS CAUSES LOSS of cellular water, cell shrinkage, elevated intracellular ionic strength, and macromolecular crowding (7). In vitro studies using simple mixtures of proteins, solutes, and artificial crowding agents have shown that high concentrations of inorganic ions, such as K⁺, Na⁺, and Cl⁻, destabilize protein secondary structure and disrupt enzyme activity (55) and that macromolecular crowding promotes nonnative protein-protein interactions, which can lead to protein aggregate formation (16, 37, 40, 64). Similar protein damage is generally assumed to occur in vivo during hypertonic stress, but this has not been demonstrated for intact animal cells, which consist of complex heterogeneous mixtures of macromolecules and volume-limited compartments and express active protein homeostasis mechanisms that can dramatically impact protein stability and aggregation.

Misfolded proteins have abnormal activity, which can disrupt cellular homeostasis (55). Protein aggregates can cause cell injury and death by functioning as sinks for properly folded and functioning proteins (22, 31) and by interacting with and damaging cell and organelle membranes (58). Cells employ numerous mechanisms to maintain their complement of properly folded and functioning proteins and to prevent and eliminate nonnative protein-protein interactions. The cellular protein homeostasis machinery functions to correctly fold proteins and to detoxify and degrade damaged proteins and nonnative protein aggregates (31, 57). During prolonged exposure to hypertonic conditions, cells express molecular chaperones (7) and accumulate organic osmolytes (61), which stabilize protein structure, inhibit nonnative protein interactions, and balance environmental osmolality. However, molecular chaperone expression and organic osmolyte accumulation occur over a period of several hours (1, 7, 11), and the fate of proteins exposed to acute cell shrinkage is unknown.

We have conducted a series of studies exploiting the genetic and molecular tractability of the nematode Caenorhabditis elegans to define hypertonic stress responses in animal cells. C. elegans is a free-living nematode that inhabits surface soil and decaying organic matter and is normally exposed to and can survive considerable osmotic stress (11, 33). During prolonged exposure to hypertonic conditions, C. elegans accumulates the chemical chaperone and organic osmolyte glycerol (33). Recently, we demonstrated that RNA interference (RNAi) knockdown of genes involved in protein synthesis and proteasomal degradation activates a pathway for glycerol synthesis, suggesting that stress-induced protein damage may act as a signal for activation of hypertonic stress-response mechanisms (34).

In this study, we used genome-wide RNAi screening to identify genes required for survival of C. elegans during acute hypertonic stress. Knockdown of 40 genes reduced survival, a phenotype we term HypertOnic Sensitive (Hos). Half of these genes function to sort, transport, or degrade proteins. They encode components of endosomal sorting complexes, lysosomes, and proteasomes. Using a fluorescent polyglutamine reporter protein, we demonstrate that cell shrinkage causes rapid protein aggregation and that endosomal sorting complexes and lysosomes function to prevent this aggregation. High concentrations of urea, a strong protein denaturant, do not cause aggregation, suggesting that factors such as macromolecular crowding contribute to protein damage during cell shrinkage. Our studies are the first to demonstrate that hypertonic stress induces protein damage in intact animal cells and that functional protein-sorting and degradation pathways are essential for survival under hypertonic conditions.
MATERIALS AND METHODS

C. elegans strains. The following strains were used: wild-type N2 Bristol, GR1373 erti-1[eg66]/IV, AM134 rmls126[\textit{Punc-54::Qo::YFP}], AM138 rmls130[\textit{Punc-54::Q24::YFP}], and AM140 rmls132[\textit{Punc-54::Q35::YFP}]. Unless noted otherwise, worms were cultured at 20°C using standard methods (5).

Genome-wide RNAi screening. Genome-wide RNAi screening was performed by combination of the 11,699 genes represented in the C. elegans ORFeome RNAi feeding library (Open Biosystems, Huntsville, AL) with an additional 6,978 genes present in the original genomic RNAi feeding library (Geneservice, Cambridge, UK). RNAi feeding bacteria were grown in 24-well control (i.e., 51 mM NaCl) nematode growth medium (NGM) agar plates, and double-stranded RNA (dsRNA) synthesis was induced using 0.2% (w/v) bacterial feeding bacteria. Bacteria with plasmid pPD129.36 were used as a control for nonspecific RNAi effects. This control plasmid expresses 202 bases of genomic RNA that are not homologous to any predicted C. elegans genes. Worms were considered alive if they displayed muscle contractions in response to repeated prodding with an eyelash. Images of individual worms were obtained with a Zeiss Stemi SV11 microscope (Chester, VA) fitted with a CCD-100 DAGE-MTI camera (Michigan City, IN). Worm volume was measured as described previously (10).

Protein aggregation assays. Synchronized larval stage 1 (L1) transgenic worms expressing a polyglutamine-yellow fluorescent protein (YFP) fusion protein (\textit{Q55::YFP}) (38) in their body muscle cells were mounted on 2% agar pads on glass slides and imaged using a LSM510-Meta confocal microscope and a Plan-Neofluar ×40/1.3 NA oil objective lens (Carl Zeiss MicroImaging, Thornwood, NY). Fluorescence recovery after photobleaching (FRAP) analysis was performed by bleaching regions of body wall muscle cells with 50 scans of a 488-nm laser at 100% power. Images were obtained every 5 s for up to 30 s after photobleaching.

Hypertonic stress assays. To measure survival, we transferred 5–8 populations of 20–80 L3-to-young adult worms to 51 or 400 mM NaCl agar, and survival was scored 1–2 days later. The screen identified 40 genes that, when knocked down, consistently decreased survival (Table 1, Fig. 1, B and C). The phenotype of these animals was termed Hos, and we refer to Hos genes as those that, on knockdown by RNAi, induce this phenotype. The Hos phenotype is characterized by immobilization of worms and acquisition of a rod-shaped morphology (Fig. 1B).

RESULTS

Genes essential for survival during hypertonic stress. As shown in Fig. 1A, C. elegans survives direct transfer from 51 mM NaCl agar to 400 mM NaCl agar. Acute hypertonic stress causes worms to lose up to 40% of their body volume (10, 11, 33). With prolonged hypertonic stress, worms slowly recover their volume (10, 11) and accumulate the organic osmolyte glycerol (33, 34). Water loss with associated cell shrinkage increases intracellular ionic strength and macromolecular crowding, which presumably cause protein damage that must be removed and repaired in order for animal cells to survive in hypertonic environments (7).

To identify genes and processes required for damage repair and survival during hypertonic stress in \textit{C. elegans}, we performed a genome-wide RNAi screen using a bacterial feeding library covering ~96% of predicted worm genes (28, 50). Eggs of an erti-1 RNAi-hypertensive strain (29) were placed on dsRNA-producing \textit{Escherichia coli} on NGM agar. Control worms were fed a bacterial clone expressing 202 bases of dsRNA that are not homologous to any predicted \textit{C. elegans} genes (plasmid pPD129.36) to account for any general, nonspecific effects of activating the RNAi pathway. After 2.5 days, worms were transferred to 400 mM NaCl agar, and survival was scored 1–2 days later. The screen identified 40 genes that, when knocked down, consistently decreased survival (Table 1, Fig. 1, B and C). The phenotype of these animals was termed Hos, and we refer to Hos genes as those that, on knockdown by RNAi, induce this phenotype. The Hos phenotype is characterized by immobilization of worms and acquisition of a rod-shaped morphology (Fig. 1B).

RNAi in \textit{C. elegans} is highly efficient and reported to recapitulate the phenotypes of 63–92% of loss-of-function mutations (28, 56) with a false positive rate of <1% (28). Using real-time RT-PCR, we found that dsRNA feeding reduced target mRNA levels by >80% for the representative Hos genes \textit{cdc-48.2} and \textit{rpn-3} (\textit{n} = 4, \textit{P} < 0.0001). These results and the reproducibility of the Hos phenotype induced by dsRNA feeding (Fig. 1) demonstrate high efficiency of gene knockdown. Gene targeting by RNAi in \textit{C. elegans} has also been demonstrated to be highly sequence specific (28, 56). Sequence analysis of the 40 Hos gene dsRNA clones predicts only six potential off-target genes. Five of six of the predicted off-target genes are paralogs of the targeted Hos genes that have redundant or similar functions (Table 2).

Hos genes function in 11 diverse cellular processes (Table 1), including cytoskeletal function, extracellular matrix formation, metabolism, protein degradation, protein sorting and
transport, signaling, and solute transport. Interestingly, 50% (20 of 40) of Hos genes are predicted to function in protein sorting, transport, and degradation (Table 1). These genes encode several components of lysosomes and proteasomes, two organelles that degrade damaged proteins, and proteins that sort and deliver substrates to these organelles. We observed slow growth, late larval arrest, and sterility with RNAi of many of the protein transport and degradation genes, which is consistent with previously reported RNAi phenotypes (www.wormbase.org). We also observed some lethality with prolonged RNAi of these genes, but this occurred well beyond the time at which they died on 400 mM NaCl. The use of first-generation RNAi worms allowed us to compare lethality of late larval or young adult worms on control and 400 mM NaCl agar.

Figure 1, B and C, shows the effect of knockdown of a subset of highly conserved protein-sorting and degradation genes on survival of N2 Bristol worms during hypertonic stress. RNAi of these genes reduced survival on 400 mM NaCl agar by 20–90%, but did not significantly ($P > 0.05$) affect survival on normal growth medium over the same time period. Seven Hos genes encode subunits of vacuolar H$^+$-ATPases (26). RNAi of vha-8 reduced survival ∼90% under hypertonic conditions (Fig. 1C). vps-4 encodes an AAA-type ATPase required for sorting of ubiquitinylated membrane proteins in late endosomes, and hgrs-1 encodes a protein that recognizes ubiquitinylated membrane proteins in early endosomes (26). Knockdown of these genes reduced survival by 80–90% (Fig. 1C). The dramatic effect of knockdown of endosomal sorting and lysosomal genes on survival in hypertonic environments indicates that detection and degradation of damaged proteins by lysosomes are mechanisms that are essential for the hypertonic stress response.

Each 26S proteasome consists of a single 20S proteolytic core and two 19S regulatory units that recognize and unfold polyubiquitinylated substrates (12). Our screen identified genes that encode four 20S and three 19S proteasome subunits (Table 1). RNAi of the 19S subunit encoding gene rpn-3 reduced survival on 400 mM NaCl agar by ∼65% (Fig. 1C), suggesting that degradation of damaged proteins by proteasomes is also an important hypertonic stress-response mechanism.

cdc-48.2 encodes an AAA-type ATPase required for transport of damaged proteins from the endoplasmic reticulum to the cytosol for degradation by proteasomes (39). RNAi of cdc-48.2 also targets a functionally redundant paralog, cdc-48.1 (39). phi-32 encodes a ubiquitin-like protein. Knockdown of these genes reduced survival by 20–30% (Fig. 1C), suggesting that hypertonic stress damages proteins in the endoplasmic reticulum and that these proteins must be transported to the cytosol for degradation by proteasomes (39). The results also suggest that proteins damaged by hypertonic stress are ubiquitinylated, so they can be targeted for degradation. Taken together, data shown in Fig. 1C and Table 1 are consistent with the hypothesis that hypertonic stress causes global protein damage and that damaged proteins must be destroyed by proteasomes and lysosomes in order for cells and organisms to survive acute exposure to hypertonic conditions.

Hypertonic stress increases ubiquitin conjugates. Ubiquitin is a highly conserved small regulatory protein that targets proteins for degradation by lysosomes and proteasomes (12). Oxidative stress and desiccation are known to increase the total level of ubiquitinylated proteins in mammalian (53) and plant cells (45), respectively. To test whether global protein ubiquitinylination is altered by hypertonic stress in C. elegans, we performed Western blot analysis of total protein lysates with a monoclonal antibody against ubiquitin. As shown in Fig. 2, high-molecular-weight ubiquitin conjugates increased 2.2-fold within 3 h after exposure of worms to 400 mM NaCl.

Hypertonic stress causes protein aggregation. Almost nothing is known about the effects of hypertonicity on proteins in animal cells. Proteins that contain contiguous repeats of glutamine residues have disordered structures, aggregate spontaneously, and have been used to identify genes, cellular processes, and physiological conditions that influence protein folding and aggregation in several systems (9, 62), including C. elegans (38).
To determine whether hypertonic stress promotes protein aggregation in animal cells, we used a transgenic strain of *C. elegans* expressing a polyglutamine-YFP (Q35::YFP) in its muscle cells (38). Q35::YFP is normally soluble and uniformly distributed throughout the muscle cell cytoplasm until worms are 4 days old, after which the protein slowly aggregates with age (38). We observed 2-day-old Q35::YFP-expressing worms to 400 mM NaCl agar and quantified YFP aggregates 1 day later. As shown in Fig. 3, the threshold for aggregation was 24–35 consecutive glutamines.

To confirm that the change in YFP distribution under hypertonic conditions reflected protein aggregation, we carried out FRAP analysis, which has been used previously in *C. elegans* to distinguish soluble vs. insoluble proteins (30, 38). Figure 4A shows a series of fluorescence micrographs that illustrate Q35::YFP distribution in body wall muscle cells of *C. elegans*.

Table 1. Gene knockdowns that cause a Hypertonic Sensitive (Hos) phenotype

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<th>Process</th>
<th>Cosmid No.</th>
<th>Gene</th>
<th>Description</th>
<th>Agg</th>
<th>Ref.</th>
<th>Present Study†</th>
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<td>CYSK</td>
<td>W07B3.2</td>
<td>gei-4</td>
<td>Intermediate filament regulation</td>
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<td>ECM</td>
<td>F45G2.5</td>
<td>bli-5</td>
<td>Protease inhibitor that affects integrity of cuticle</td>
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<td>col-58</td>
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<td>F13D12.6</td>
<td>Serine carboxypeptidases, lysosomal cathepsin A</td>
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<td>PDP</td>
<td>CD4.6</td>
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CYSK, cytoskeleton; ECM, extracellular matrix; MET, metabolism; NCAD, nucleic acid binding or modification; PD, protein degradation; PDL, protein degradation by lysosomes; PDP, protein degradation by proteasomes; PST, protein sorting and transport; SG, signaling; ST, solute transport; U, unknown; LCFA, long-chain fatty acid; ER, endoplasmic reticulum; PFG, fibrolast growth factor; Agg, suppressor of polyglutamine aggregation. Genes were confirmed by sequencing of RNA interference (RNAi) plasmids from positive bacterial clones. *RNAi clones with potential off-target genes (Table 2).

To determine whether hypertonic stress promotes protein aggregation in animal cells, we used a transgenic strain of *C. elegans* expressing a polyglutamine-YFP (Q35::YFP) in its muscle cells (38). Q35::YFP is normally soluble and uniformly distributed throughout the muscle cell cytoplasm until worms are 4 days old, after which the protein slowly aggregates with age (38). We exposed 2-day-old Q35::YFP-expressing worms to high-NaCl agar and quantified YFP aggregates 1 day later. As shown in Fig. 3, the threshold for aggregation was 24–35 consecutive glutamines.

To confirm that the change in YFP distribution under hypertonic conditions reflected protein aggregation, we carried out FRAP analysis, which has been used previously in *C. elegans* to distinguish soluble vs. insoluble proteins (30, 38). Figure 4A shows a series of fluorescence micrographs that illustrate Q35::YFP distribution in body wall muscle cells of worms exposed to agar containing 51 or 400 mM NaCl. The time course of fluorescence recovery is shown in Fig. 4B. Approximately 80% of the fluorescence recovered within 30 s after photobleaching in worms exposed to 51 mM NaCl. In contrast, worms exposed to 400 mM NaCl exhibited <10% fluorescence recovery during the same time period. These results are consistent with the localization of Q35::YFP to aggregates where individual proteins are immobilized and unable to diffuse into the bleached area.

Cell shrinkage selectively induces rapid protein aggregation. To determine whether the rapid protein aggregation we observed was selectively induced by high NaCl, we quantified the number of Q35::YFP aggregates formed over time follow-
ing acute exposure to NGM agar supplemented with osmotically equivalent levels of NaCl (349 mM), sorbitol (611 mM), or urea (697 mM). Aggregation increased rapidly over the first few hours of exposure to 400 mM NaCl or 611 mM sorbitol (Fig. 5A). In contrast, 697 mM urea had no effect on Q35::YFP aggregation (Fig. 5A).

Oxidative stress promotes aggregation of polyglutamine-containing proteins (20). Exposure to hypertonic medium elevates reactive oxygen species in cultured mammalian cells (32, 63). To determine whether oxidative stress could be responsible for the protein aggregation we observed, we exposed

### Table 2. Potential off-target genes

<table>
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<th>Targeted Cosmid No.</th>
<th>Targeted Gene</th>
<th>Potential Off-Target Genes</th>
<th>Off-Target Process</th>
<th>Off-Target Gene Description</th>
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<td>W05G11.3</td>
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<td>PD</td>
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<td>F52C6.3</td>
<td>phi-32</td>
<td>math-32</td>
<td>SG</td>
<td>Meprin-associated Traf homology domain containing</td>
<td>No</td>
</tr>
<tr>
<td>C41C4.8</td>
<td>cdc-48.2</td>
<td>cdc-48.1</td>
<td>PST</td>
<td>AAA-type ATPase, ER protein sorting</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Potential off targets were identified with the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

Oxidative stress promotes aggregation of polyglutamine-containing proteins (20). Exposure to hypertonic medium elevates reactive oxygen species in cultured mammalian cells (32, 63). To determine whether oxidative stress could be responsible for the protein aggregation we observed, we exposed
worms to 10 mM paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride). Paraquat is a redox cycling compound that causes oxidative stress in C. elegans (2, 18). As shown in Fig. 5A, paraquat did not induce formation of Q35::YFP aggregates, suggesting that factors other than oxidative stress alone contribute to protein aggregation under hypertonic conditions. Urea is a well-known protein denaturant. In addition, it is a small nonpolar solute that readily permeates cell membranes and, therefore, may induce little or no water loss from cells and organisms (23, 55). The data in Fig. 5A thus suggest that water loss may be responsible for the rapid protein aggregation in high-NaCl and high-sorbitol media. To test this possibility, we measured total body volume in worms exposed to osmotically equivalent amounts of NaCl, sorbitol, or urea. As shown in Fig. 5B, worms exposed to NaCl or sorbitol lost ~30% of their volume after 0.5 h and then, over the following 2–3 h, recovered ~50% of their lost volume. Conversely, worms exposed to urea lost only ~19% of their volume after 0.5 h and then, within 1 h, fully recovered the lost volume. Taken together, the data in Fig. 5 suggest that cell shrinkage caused by high concentrations of nonpermeable osmolytes promotes rapid protein aggregation.

Interestingly, after 24 h, the number of Q35::YFP aggregates formed in worms exposed to high NaCl was nearly double that in animals exposed to high sorbitol (29 ± 4 vs. 15 ± 6, n = 6–8 worms). We showed previously that osmotically equivalent amounts of NaCl and sorbitol cause similar degrees of water loss in C. elegans but that high NaCl causes a greater reduction in survival (10). It is likely that worms exposed to high NaCl have an increased salt load, which results in elevation of extracellular and intracellular ionic strength beyond that induced by osmotic water loss alone.

Hos genes prevent protein aggregation. The results of our RNAi screen and Q35::YFP studies indicate that cell shrinkage promotes protein aggregation and that functional proteasomes and lysosomes are required for survival during acute hypertonic stress. This suggests that proteins damaged by hypertonic stress must be cleared from the cell to prevent aggregation and ensure survival. If this hypothesis is correct, then many Hos genes should play a role in preventing accumulation of damaged proteins under normal physiological conditions.

Using Q35::YFP-expressing worms, Nollen et al. (43) identified 187 genes by RNAi screening that function to slow aging-induced protein aggregation. We found that 9 of our 40 Hos genes overlap with this gene set (Table 1), which is a
cell shrinkage promotes protein aggregation

To further examine the role of Hos genes in preventing aggregation, we quantified the number of Q35::YFP aggregates in nonosmotically stressed 3-day-old worms fed E. coli-producing dsRNA to vha-8, vps-4, hgrs-1, rpn-3, cdc-48.2, and phi-32. RNAi of vha-8, vps-4, hgrs-1, rpn-3, and cdc-48.2 caused a significant (P < 0.05) increase in the number of Q35::YFP protein aggregates in worms grown on normal (51 mM NaCl) agar (Fig. 6, A and B).

We also quantified the effect of RNAi of these genes on aggregate formation in worms grown on 51 mM NaCl agar for 2 days and then transferred to 300 mM NaCl agar for 1 day (Fig. 6, A and B). This level of hypertonic stress alone did not cause a significant (P > 0.3) increase in Q35::YFP aggregation, but it did cause a significant (P < 0.02) increase in aggregation with RNAi of vha-8, vps-4, and hgrs-1, genes that function in endosomes and lysosomes (Fig. 6B). These results demonstrate that endosomal sorting complexes and lysosomes function to prevent accumulation of these stress-induced aggregates.

As shown in Fig. 6C, there is a highly significant (P < 0.01) inverse correlation between survival and the number of protein aggregates resulting from RNAi of the six Hos genes tested during hypertonic stress, suggesting that prevention of protein aggregation is an essential mechanism for survival in hypertonic environments. Interestingly, vha-8, vps-4, and hgrs-1, the three genes required for endosomal and lysosomal function, had the largest impact on survival and protein aggregation. These results suggest that endosomes and lysosomes play a critical role in cell survival by preventing accumulation of hypertonicity-induced protein aggregates.

Protein aggregation is not induced by acute inhibition of protein degradation pathways. Given that protein degradation pathways prevent age-induced (43) (Table 1) and hypertonicity-induced (Fig. 6) protein aggregation, it is possible that hypertonicity causes protein aggregation by acutely inhibiting protein degradation. To test this possibility, we compared the rate of Q35::YFP aggregate formation in worms exposed to hypertonicity and in worms treated with inhibitors of protein degradation. Lysosomal function was inhibited with 20 mM chloroquine, a widely used lysosomotropic amine that inhibits proteases by raising lysosomal pH (14). Proteasomal function was inhibited with 100 μM MG-132, a cell-permeable substrate analog and potent translocation state inhibitor of protease activity (35). As shown in Fig. 7, treatment of worms with both chloroquine and MG-132 did not cause significant (P > 0.05) protein aggregation during the 24-h exposure period. In contrast, protein aggregation was observed 3 h after exposure of worms to 400 mM NaCl (Fig. 7A).

To verify that chloroquine inhibits lysosomal function in C. elegans, we measured the fluorescence of acridine orange (13), a dye that accumulates in acidic lysosomal compartments in worms (26, 44). Exposure to 20 mM chloroquine for 3 h decreased acridine orange fluorescence by 74 ± 0.04% (P < 0.0001, n = 264), demonstrating robust alkalization of lysosomal pH.

MG-132 has been shown to inhibit proteasomal degradation in C. elegans (46), but the time course of inhibition is unknown. We therefore also tested the effect of proteasomal inhibition by RNAi of rpn-3, which causes a fully penetrant larval arrest phenotype common to proteasomal disruption in worms (50). Q35::YFP-expressing worms were fed rpn-3 dsRNA for 2 days, starting with synchronized L1 larvae. At the beginning of the 3rd day of dsRNA feeding, they were also exposed to 20 mM chloroquine. As shown in Fig. 7, no significant (P > 0.05) increase in protein aggregation was
observed during the first 9 h of chloroquine treatment in *rpm-3* RNAi worms. Significant protein aggregation was observed in *rpm-3* RNAi worms 24 h after exposure to chloroquine. However, the number of aggregates was 2.6-fold (*P* < 0.002) lower than in hypertonically stressed animals (Fig. 7).

Taken together, data in Fig. 7 demonstrate that hypertonicity causes protein aggregation much more rapidly than inhibition of lysosomal and proteasomal function. This conclusion is supported by studies in cultured mammalian cells, which demonstrate that protein aggregation is induced only after prolonged inhibition of proteasome degradation (3, 20). Although we cannot completely rule out the possibility that compromised protein degradation contributes to protein aggregation during hypertonic stress, our results indicate that other more rapidly acting mechanisms, such as macromolecular crowding (16), induce aggregation in hypertonically stressed cells.

**Acclimation to hypertonic environments activates pathways that prevent protein aggregation.** We previously demonstrated that acclimation of *C. elegans* to mild hypertonic stress (200 mM NaCl) activates pathways that dramatically increase survival during exposure to more extreme hypertonicity (33). Given that survival and prevention of protein aggregation are highly correlated (Fig. 6C), we postulated that acclimation to mild hypertonicity might promote survival by activating pathways that prevent hypertonicity-induced protein aggregation. As shown in Fig. 8, acclimation of worms to 200 mM NaCl dramatically suppressed aggregate formation compared with worms grown on 51 mM NaCl agar.

**DISCUSSION**

We performed a genome-wide identification of genes required for osmotic stress resistance in *C. elegans*. Our studies demonstrate that hypertonic stress disrupts protein homeostasis and that functional protein degradation pathways are essential for survival in hypertonic environments. Data in Figs. 3–5 demonstrate that hypertonicity-induced cell shrinkage accelerates aggregation of a polyglutamine-containing protein. Cell shrinkage increases intracellular ionic strength and causes macromolecular crowding (7, 55). Both of these effects may lead to increased protein aggregation.

Crowding is expected to have a universal aggregation-promoting effect by increasing the concentration of all cellular proteins (64). *C. elegans* can lose ≥40% of its volume during hypertonic stress (10, 11, 33). Approximately 30–40% of a cell’s normal volume can be occupied by macromolecules (19, 65). Thus cellular proteins are in a severely confined state that can promote diverse nonnative interactions that lead to aggregate formation (16, 37, 40, 64). Cell shrinkage further confines macromolecules and, thereby, enhances the propensity for protein aggregation.

High ionic strength is a well-known disruptor of protein secondary structure that can increase protein aggregation (23, 55). Studies with yeast and in vitro systems suggest that high ionic strength also disrupts initiation and elongation steps of protein synthesis, resulting in incomplete and immature polypeptides that may be prone to aggregate (6, 59).

Our findings with Q35::YFP likely reflect a general effect of cell shrinkage on protein aggregation. Many proteins aggregate under crowded and denaturing conditions, and the mechanisms of aggregation are thought to be universal (31, 58). In particular, proteins with high densities of glutamine, or the structurally related polar residue asparagine, have a high propensity for aggregation because of strong hydrogen bonding (36). Q/N-rich domains occur more commonly in the proteomes of eukaryotes, including *C. elegans*, than expected by random chance, suggesting that they are maintained by natural selection (36). It has been suggested that Q/N-rich domains normally function to mediate specific protein-protein interactions and that aggregation is an extreme manifestation of this function that is enhanced by mutation, aging, and stress (36, 48).

Many studies have demonstrated that protein degradation pathways function to modify the formation and toxicity of age-induced and spontaneous protein aggregates (3, 4, 17, 27, 43, 51). The data in Fig. 6 suggest a prominent role for endosomal protein-sorting complexes and the lysosome in prevention of protein aggregation and death during cell shrinkage. Recent studies have demonstrated that *Drosophila* and...
human endosomal protein-sorting complexes, which include orthologs of vps-4 and hgrs-1, function to reduce protein aggregates via macroautophagy (17, 52). During macroautophagy, substrates such as protein aggregates or aggregate intermediates are engulfed in a double membrane to form autophagosomes, which fuse with endosomes and mature into autolysosomes, where proteins are degraded (8). Endosomal protein-sorting complexes are thought to mediate the latter fusion and maturation steps (52). The importance of this process in preventing accumulation of protein aggregates is highlighted by frontotemporal dementia and amyotrophic lateral sclerosis, which are diseases caused by mutations in CHMP2B, an endosomal protein-sorting component (47, 54).

We hypothesize that a similar mechanism, mediated by vps-4 and hgrs-1 (Fig. 6, A and B), functions in C. elegans to reduce protein aggregates during hypertonic stress.

We propose a model in which cell shrinkage promotes nonnative protein-protein interactions via macromolecular crowding and elevated intracellular ionic strength (Fig. 9). Protein aggregates or aggregation intermediates may be sorted through endosomes and degraded in lysosomes or ubiquitylated and degraded by the 26S proteasome, two well-known pathways that inhibit protein aggregation (17, 43, 52). If these protein-sorting, transport, and degradation pathways are compromised, or if the amount of nonnative protein-protein interactions exceeds the capacity for degradation, then aggregates form and contribute to cell death.

Surprisingly, our screen did not identify heat shock proteins (HSPs) or enzymes known to function in organic osmolyte accumulation as essential for survival during acute hypertonic stress. Organic osmolytes and HSPs function as chemical and molecular chaperones, respectively, and both are thought to promote cell survival by preventing protein misfolding and by functioning to refold denatured proteins (7, 57, 61). Some HSPs also function to recruit misfolded proteins to CHIP, an E3 ubiquitin ligase that regulates degradation of damaged proteins (41). We showed previously that genetic disruption of the synthesis of glycerol, which is the dominant organic osmolyte in C. elegans, does not decrease short-term survival of adults in hypertonic environments. Instead, inhibition of glycerol synthesis slows development and causes sterility (34).

Accumulation of glycerol in C. elegans is a slow process and occurs over a period of many hours after worms are exposed to hypertonicity (33). The present studies, along with our previous work, suggest that organic osmolytes likely promote survival during long-term, rather than acute, hypertonic stress.

HSP expression in mammalian cells is induced by hypertonic stress (7). The genome of C. elegans encodes ≥40 HSPs (www.wormbase.org). The failure of our RNAi screen to detect HSPs may reflect functional redundancy. Alternatively, similar to glycerol accumulation, HSP expression can take hours to fully activate (7, 33). Thus HSPs may be important for limiting protein damage during prolonged exposure to hypertonic conditions.

Chemical and molecular chaperone pathways are known to be induced by mild hypertonic stress in diverse organisms and cell systems (7, 11). Our demonstration that acclimation to mild hypertonic conditions dramatically suppresses polyglutamine aggregation during extreme hypertonic stress (Fig. 8) suggests that protein degradation pathways may be upregulated by hypertonic stress. It will be important to determine if and how such upregulation occurs.

We recently demonstrated that, in the absence of hypertonic stress, RNAi inhibition of genes involved in RNA processing, protein translation, cotranslational protein folding, and proteosomal degradation of proteins activates a pathway regulating synthesis of the organic osmolyte glycerol (34). Disruption of protein synthesis, folding, and degradation is expected to cause cytoplasmic accumulation of damaged proteins. We have postulated that organic osmolyte accumulation pathways may be activated specifically by osmotically induced disruption of new protein synthesis and cotranslational folding, rather than by damage to fully functional proteins (11, 34). Such a mechanism would allow cells to discriminate between osmotically induced and other forms of stress-induced protein damage.

The present study expands on our previous findings and underscores the critical importance of protein homeostasis in the cellular osmotic stress response. Damaged proteins may not only function as signals that activate osmotic stress response pathways, but the rapid detection and removal of misfolded and aggregated proteins are essential mechanisms required for survival of hypertonically stressed cells. Importantly, knockdown of genes required for endosomal sorting and lysosome function does not activate the glycerol synthesis pathway (34). However, endosomes and lysosomes do play a prominent role in preventing polyglutamine protein aggregation and in promoting survival during hypertonic stress (Fig. 6). This indicates that the types of protein damage that cause aggregation and cytotoxicity may not be the same as those that activate glycerol synthesis.

Fig. 9. Model of protein homeostasis during hypertonic stress. Hypertonicity causes osmotic water loss and cell shrinkage. Increased macromolecular crowding and protein denaturation induced by elevated intracellular ionic strength promote nonnative interactions between proteins, which can lead to aggregation. Accumulation of protein aggregates can inhibit enzyme complexes, damage membranes, and lead to cell death. Endosomal sorting complexes, lysosomes, and the proteasome function to prevent accumulation of cell shrinkage-induced protein aggregates.
The effects of cell shrinkage on protein aggregation that we observed in C. elegans are likely to be similar for any cell that is exposed to significant hypertonic stress. Cells in the mammalian renal medulla are exposed to large shifts in extracellular osmolality that accompany changes in water excretion (42). Protein aggregation disrupts cellular homeostasis and is associated with over 20 human diseases (31, 57, 58). Although most symptoms of protein aggregation-associated diseases are observed in the kidneys of mammals, where hypertonic stress could accelerate protein aggregate formation and cell injury (15, 24). The importance of organic osmolyte accumulation to renal medullary cell survival during hypertonic stress is well documented (7, 25). It will be important to examine the role of protein damage and degradation in renal medullary cell survival as well as in organisms that inhabit osmotically unstable environments.

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REFERENCES


